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Imunohistochemical profile of the neuroblasts of the peripheral sympathetic nervous system and human neuroblastoma of childhood

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Chapter 1

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1.1 Embryological development of peripheral sympathetic nervous system (PSNS)

The development of the human peripheral sympathetic nervous system (PSNS) originates from neuroectodermal cells of the neural crest, bilateral structures that are formed during the process of closing the tube neural, which are detached from the longitudinal edges to form. The neural crest is derived from the dorsal neural tube and extends all along the spinal cord and hindbrain. Thus, when the neural crest cells begin their migration they bring with them the information related to their site of origin, including those the expression of several genes. Regardless of their site of origin, all neural crest cells must undergo a state of transition for access to begin their migration. All these cells begin as neuroepithelial cells and as such possess all the intercellular junctions and adhesive interactions that serve to keep the epithelial cells in position. To move, the neural crest cells should reduce the expression of the genes for these adhesive proteins and undergo a transition from epithelial cells to mesenchymal cells (the first being cohesive to form a layer and the second more weakly bound and with high tendency to migrate freely). Therefore, the neural crest cells express different transcription factors, including snail1 and snail2 that repress the expression of proteins junctional intercellular adhesion molecules and epithelial. After gaining their ability to migrate, the neural crest cells reach their final destination, and cease to express Snail and other transcription factors that promote the "mesenchymal

condition" required for migration. It is thought that this change is the result of the integration of numerous signals that the neural crest cells encounter along their migration routes. Very early (4th - 5th week of development), the plates are fragmented in primitive sketches ganglion spinal (future dorsal root ganglia) and the nests of neuroblasts undifferentiated (NNI), interconnected by thin nerve fibers consist of axons and Schwann cells.

The cells constituting the neural crest elements are capable pluripotent, that is multidirectional differentiation: nerve (ganglion cells), glial (Schwann cells), neuroendocrine (chromaffin cells or neuroendocrine) and melanocytic. In the earliest stages, in addition to cells already "committed" to a cell line well defined, there are cells with dual differentiation capacity (eg, nerve and glial cells), as well as cells "committed" capable of "transdifferentiation" that is able to transform the their phenotype in another (for example, a nerve cell can differentiate into a cell neuroendocrine and vice versa).

In human embryos of 5-6 weeks of development, one can observe numerous NNI consisting of undifferentiated neuroblasts, ie cells that have, overall, a lymphocyte-like appearance. In fact they have a round shape, round and hyperchromatic nuclei containing small nucleoli (Fig. 1B, 2A, 2B) and a rhyme thin cytoplasm. These NNI migrate from regions along the paravertebral area and para-aortic pre until the adrenal gland that, at this stage, consists exclusively of the cortical component mesoderm (Fig. 1A). From undifferentiated neuroblasts, for processes of cell differentiation and maturation, will form the following structures: a) the paravertebral sympathetic ganglia that extend from the cervical region to the lumbosacral region and the sympathetic ganglia and previscerali intraviscerali b) the adrenal medulla; c) the paraganglia. The NNI that are located laterally to the spine are the primitive sketch of the paravertebral sympathetic ganglia, while others migrate ventrally to the aorta and lead in the vicinity of the viscera (gastro-intestinal tract, uro-genital, etc..), Where they form the sketches primitive of the sympathetic ganglia previscerali.

Some NNI push themselves inside the wall of many hollow organs (esophagus, stomach, small and large intestine, bladder, uterus, etc ..., where they form the nerve plexus ganglion (sympathetic ganglia intraviscerali). The NNI that are located in correspondence of the adrenals begin the invasion of the adrenal capsule (Fig. 1A, 1C) and is pushed towards the interior of the gland where,

reaching the deepest portion, initiates the formation of the adrenal medulla. Finally, some NNI that follow the nerve fibers that interconnect the various sympathetic ganglia in developing, differentiate into aggregates, rather variable in size, of neuroendocrine cells defined sympathetic paraganglia. From the 8th - 9th week of development, the NNI that will form the future chains ortosimpatiche paravertebral, and the various sympathetic ganglia peri-and intra-visceral differ cells with large eosinophilic cytoplasm and large core vesicular containing a large nucleolus: the ganglion cells. It is known from the literature that the undifferentiated neuroblasts are detectable immunohistochemically using different antibodies, including the neuron-specific enolase, BCL-2, CD44 etc.. (Table 1) (Magro et al., 1999).

1.2 Ganglionic cell lineage

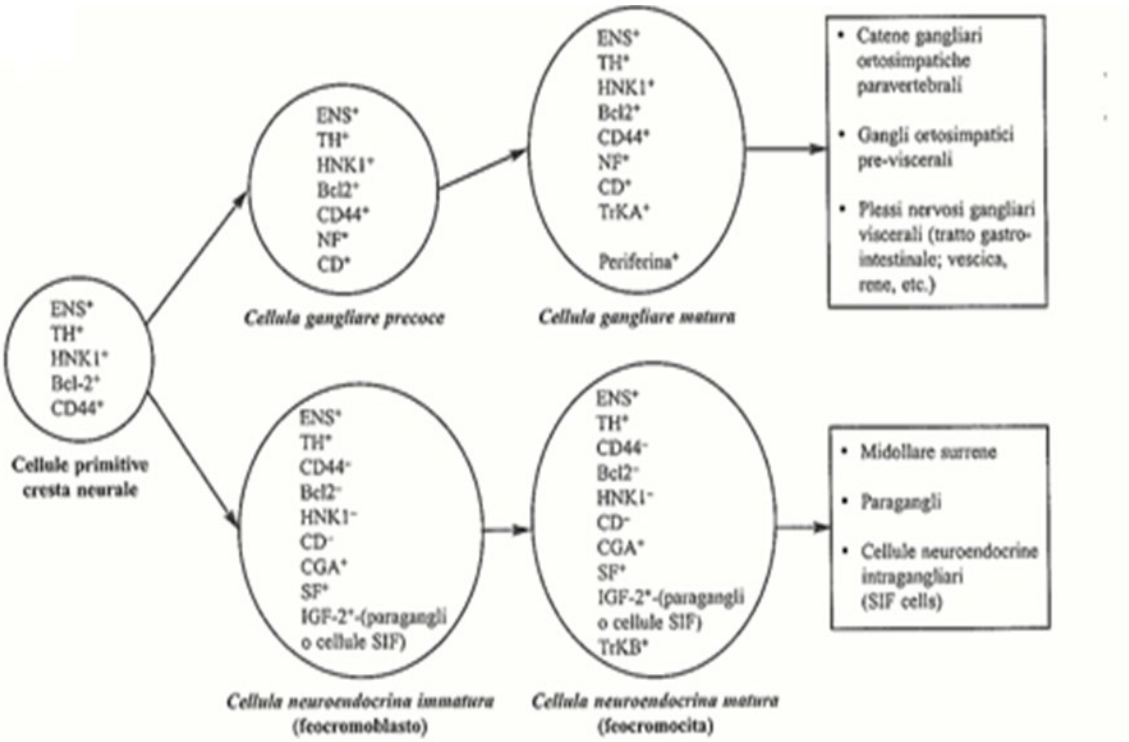
From the 7th - 9th week of development is possible to observe the differentiation of the line ganglionic; at this stage of the nests within neuroblastic is possible to identify rare cells with morphological characteristics that diverge from neuroblasts undifferentiated, for the presence of a more evident and a cytoplasm vesicular nucleus with a prominent nucleolus: the "immature ganglion cells." These fetal ganglion cells are immunoreactive to tyrosine kinase receptors for neurotrophins (TrkA and TrkC) (Tab.1). Their maturation is characterized by a progressive increase in the size of both the cytoplasm and the nucleus, which becomes increasingly vesicular and contains a single prominent nucleolus and central. The differentiation of undifferentiated neuroblasts into ganglion cells occurs mainly in those nests phones that will form the chains of the paravertebral sympathetic ganglia and sympathetic ganglia pre-and intra-visceral (Magro et al., 1999). Ganglion cells will differentiate from the nests neuroblastic intrasurrenali also, but to a quantitatively reduced. The number of ganglion cells in the later stages of the development of human SNOP remain relatively constant throughout life.

1.3 Cromaffin cell lineage

The neuroendocrine cells or cromaffin are derived from precursors neuroblastic genetically programmed to form the neuroendocrine cell line intragangliare, the sympathetic paraganglia and the adrenal medulla (Table 1). The differentiation of neuroblasts towards the line neuroendocrine can be easily highlighted immunohistochemically using antibodies anti-chromogranin A. Intraganglionic neuroendocrine cells, also known as "chromaffin cells intraganglionic" or "small cells intensely fluorescent" (SIF) cells, are formed inside of the nests of neuroblasts that will form the chains paravertebral sympathetic nervous system, and most of them disappear in the neonatal period.

The paraganglia are clusters of neuroendocrine cells, scattered in different parts of the body, which originate from the nests of undifferentiated neuroblasts arranged along the nerve fibers that interconnect the various sympathetic ganglia. Most paraganglia are macroscopic structures in axial arrangement, distributed along the pre-aortic area, from the thorax to the pelvis. Most paraganglia sympathetic fetal disappears in the neonatal period and the residual component undergoes a size reduction.

Tab.1



Magro G et al., 2000

1.4 Immunohistochemical results

Within the nests of undifferentiated neuroblasts, by methods of immunohistochemistry, protein

S-100 colors nucleus and the cytoplasm of cells that have the dendritic-like cytoplasmic structures and taking direct relationships with undifferentiated neuroblasts. These cells positive for S-100 protein, we define "sustentaculari cells." The same antibody (anti-S-100) shows Schwann-like cells that completely surround the nests of undifferentiated neuroblasts, separating them from the outside. These Schwann-like cells originate from nerve fibers that interconnetono these nests in migration. This topographic distribution of Schwann-like cells is

strategic for the processes of migration and differentiation of neuroblasts undifferentiated.

1.5 Line Glial

In human embryos and fetuses of gestational age between the 7th and the 15th week of development, using antibodies to S-100 protein we identified in SNOP human extra-and intra-visceral (especially adrenal) three cell populations glial different in morphology , topography, and relationships phones: Schwann cell-like cells and Schwann cells sustentaculari or satellites (Fig. 1A, 1C, 2A, 2B).

Schwann cells surround the axons of nerve fibers that interconnect the nests neuroblastic undifferentiated. The Schwann-like cells, spindle cells are equipped with fine and long bipolar cytoplasmic extensions, localized at the periphery of the nests of neuroblasts. These cells have relationships of continuity with the Schwann cells of the nerve fibers which, extending along the periphery of the nests neuroblastic, form a continuous cell monolayer (Fig. 1A, 1C).

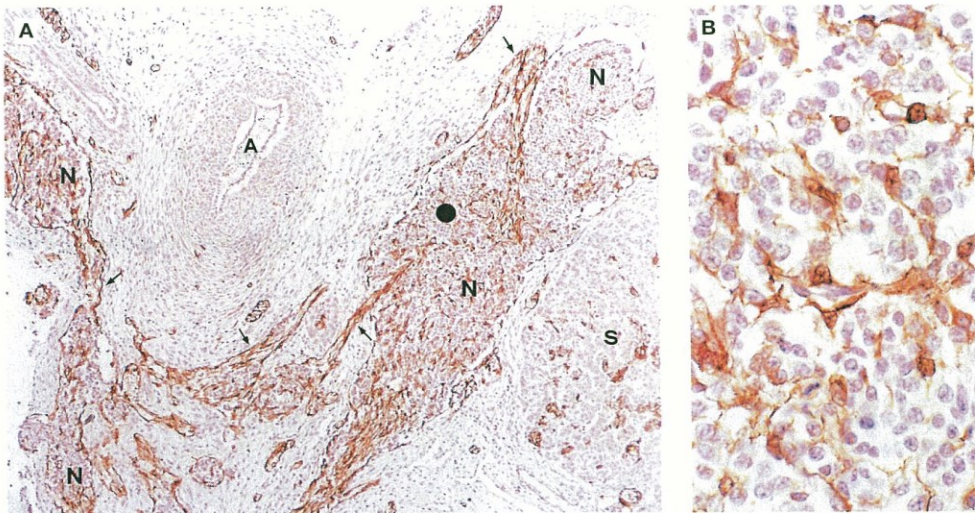
Sustentaculari cells are oval or rounded cells in the early stages of development are in direct contact, via cytoplasmic dendrites, with undifferentiated neuroblasts (Fig. 1B). In subsequent stages, contract relations with both neuroendocrine cells (Fig. 2B) that with those ganglion in developing, by surrounding them individually (satellite cells) (Fig. 2C).

1.6 Line Neuroendocrine

The adrenal medulla is the prototype of the sympathetic paraganglia and lends itself as an ideal model to study the ontogeny of human neuroendocrine cell line. In the adrenal glands of gestational age between the 7 th and 32 th week of development, several groups of nests of neuroblasts undifferentiated always interconnected by nerve fibers migrate from the cortex to the deep veins, located in the glandular portion innermost (Fig. 2A). In the period between the 12th and the 24th week of development, some nests of neuroblasts show a central cystic degeneration, with a peripheral rim of residual cell more or less extensive. At the periphery of these nests are differentiated cells that are larger than the neuroblasts, distinguished above all by their vesicular nucleus and clear cytoplasm (Fig. 2B). These cells, also known as "feocromoblasti", positive for chromogranin A, represent the early stage of the neuroendocrine line of the adrenal medulla and

organize themselves into small cell aggregates or as single cells that take direct contact with the cells of the adrenal cortex. In later stages, from 24th to 38th week of gestation, the nests of undifferentiated neuroblasts progressively decrease in number until to disappear completely in the neonatal adrenal glands. Simultaneously, we are witnessing a continuous and progressive differentiation of neuroendocrine cells that, by organizing themselves into cell cords tightly crammed together, form the adrenal medulla, now topographically distinct from the cortex. In the adrenals and in neonatal and adult, the bone marrow is constituted by neuroendocrine cells mature (feocromociti), from rare ganglion cells and from cells sustentaculari (Fig. 2C).

Fig.1

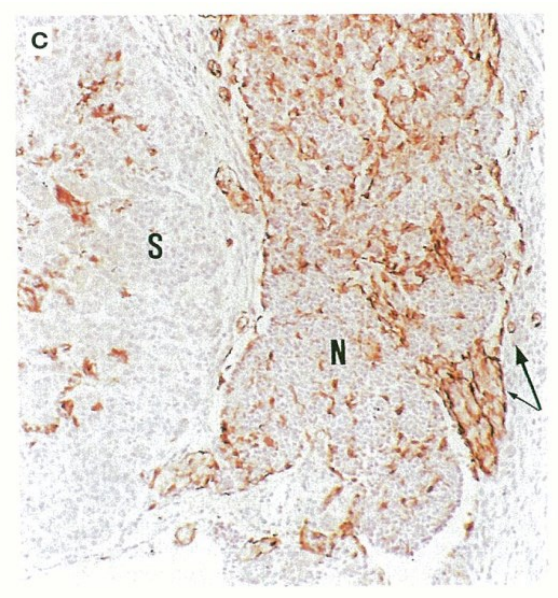


Magro G et al., 2000

Fig. 1 Human fetus of 8 weeks

A. The protein S-100 shows nerve fibers (arrows), spindle cell-like cells and Schwann sustentaculari, respectively at the periphery and inside of the nests neuroblastic (N) in position para-aortic and pre-(A) and in the vicinity of the adrenal (S). B. Higher magnification of the marked area (●). Sustentaculari cells with clear cytoplasmic dendrites in direct contact with the undifferentiated neuroblasts.

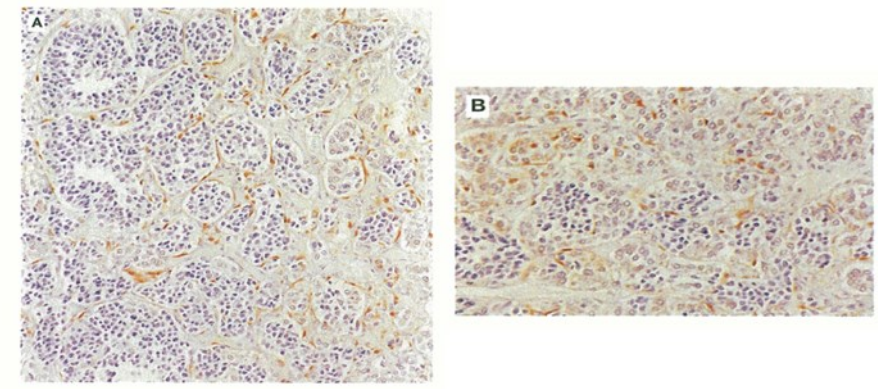
Fig.1C



Magro G et al., 2000

Fig. 1C Nests of neuroblasts (N) adhering to the adrenal gland (S). Marking with anti-S-100 protein that shows the distribution of Schwann-like cells along the periphery of the nests neuroblastic in continuity with Schwann cells.

Fig.2

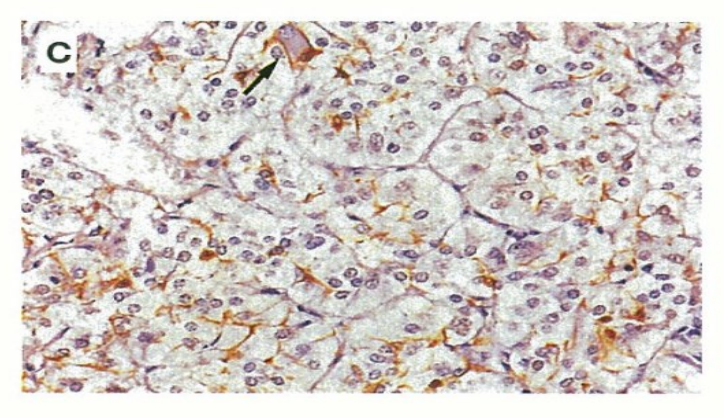


Magro G et al., 2000

Fig. 2 fetal adrenal 15-week A. In the glandular portion inner neuroblastic nests are observed surrounded by Schwann-like cells positive for S-100 protein.

B. Chromaffin cells in the process of differentiation are in direct contact with the cells sustentaculari S-100 positive.

Fig. 2C



Magro G et al., 2000

Fig. 2C adult adrenal

Among the chromaffin cells are observed only cells sustentaculari S-100 positive. Note a rare ganglion cells (arrow) surrounded individually by a cell sustentaculare.

1.7 Glial cells and neuroblastoma

Currently it is believed that neuroblastoma represents the neoplastic counterpart of immature precursor cells embryo-fetal human SNOP, whose genesis probably to be found in clonal expansion correlation to an arrest of the normal differentiation process that, in most cases, it affects the line ganglioni, but that in some cases seems to involve also the line - neuroendocrina extra- adrenal. This concept is based on the following evidence : I) generally the offices of insorgention of neuroblastoma correspond to the distribution of peripheral sympathetic nervous system structures, II) autopsy studies conducted in neonatal adrenal glands , have evidently a high incidence of small nodules neuroblastic that histologically and immunophenotypically, are similar to neuroblastomatosi nodules (histologic growth pattern type nodular or lobular, III) the differentiation undifferentiated morphology of neuroblasts towards the ganglion line cell during ontogeny reflects that observation in neuroblastoma, IV) the differenziazione/maturation in vitro

neuroblastoma that recapitulates some of osservabile ontogeny of the neural crest; V) ontogenetic expression of many molecules , including oncoproteins (Bcl -2 , c - erbB2) ,β-2 Microglobulin , enzymes lysosomiali (cathepsin D) , is similar to that observed in the different maturational stages of neuroblastoma cell; VI) neuroblastoma, as well as normally occurs in its precursor cells during embryo-fetal development, can undergo spontaneous differentiation and maturation in vivo (transformation from neuroblastoma in ganglioneuroma).

Neuroblastoma is constituted by a cell – population heterogeneous represented by undifferentiated cells lymphocyto- like, from cells that show a variable degree of differentiation and ganglionic glial cells , especially cellular the Schwann and , to a lesser extent, cell type - sustentacular. The importance of glial cells is testimonies by the fact that the classification adopted in most '80s, the Shimada Classification, subdivided all the tumor neuroblastic into two broad categories depending on the amount of stroma consists of Schwann cells : " stroma -poor or stroma -rich tumors " with a different meaning prognostic. Today, the classification most used is that Joshi et al . which divides the neuroblastoma into three subtypes: undifferentiated (neuroblasts exclusively indifferentiated), poorly differentiated (cells with differentiation ganglionic <5 %) and differentiating (cells with differentiation ganglionic > 5 %).

1.8 Localization of glial cells in neuroblastoma

The nerve cell component of neuroblastoma has tendency to grow in nests of variable size and shape, surrounded by septa fibro -vascular more or less thick (pattern nodular or lobular). Immunohistochemical studies , used antibodies anti-S-100 protein , show, in most cases, Schwann cells in the fibrovascular septa; tumor in some areas , in which the septa are very thin, the Schwann cells form a monolayer.

Glial cells in the ontogeny of the peripheral nervous system sympathetic human phone that completely surrounds the tumor nodules , with appearing as a pattern that resembles closely the observate during ontogeny in the nests neuroblastic indifference financed. In some cases of neuroblastoma know recognizable a relatively small number of cells S-100 positive in direct contact with the tumor cells , to form round or oval , with cytoplasmic dendrites , referring sustentaculari cell. Even in this case , is high you a close analogy about the topographic

distribution and reports undertaken between cells and neuroblasts sustentaculari, between the tumor pathology and ontogeny .

It is quite surprising that the fibrovascular septa pre feel in neuroblastoma contain molecules of the matrix such as the extracellular collagen type IV and VI, laminin, and fibronectin, including know not immersed Schwann cells , believed to be responsible their synthesis. All these data indicate that, in the areas neuroblastomatose with a pattern of growth of nodular type, the distribution and the relationship between Schwann cells and cellular the sustentaculari with the molecules of the extracellular matrix and neoplastic cells reflect those observed during the ontogeny of undifferentiated neuroblastic nests . This further reinforces the notion that the neuroblast but it is a neoplasm that recapitulates the ontogeny of SNOP human being, not only limited to the nervous component tumor, but also with respect to the line glial.

1.9 The origin of Schwann cells in neuroblastoma

Based on ontogenetic considerations and experiments in vitro, it was thought that the Schwann cells, as well as ganglion cells, came from a differentiation process neuroblasts cancer and would therefore be considered de rare also of neoplastic nature. Cytogenetic studies of differentiating neuroblastoma have shown, however, that the Schwann cells, as opposed to the cells neuroblastic, do not present numerical chromosomal abnormalities and which are therefore to be considered as reactive cells as cancer. This concept is widely accepted and it has been speculated that the Schwann cells of the neuroblastoma cells are cells that infiltrate the tumor by the outside (originally from the peripheral nerves adjacent?) in response to trophic signals produced by the same neurobastoma.

Although some factors were candidates for this role (glial growth factor , growth factor -β, the platelet growth factor) , has not yet been documentation their activity.

If today we share without reservation that the neuroblastoma derived from neural crest precursors, it is rational that the cells of Schwann present in this tumor may arise out glial cells normally associated with neuroblasts undifferentiated during development. A block to a maturational stage of normal path of neuroblasts ontogenetic embryo- fetal which also involves glial cells, which are present during this event , becoming the glial component of the tumor. Our hypothesis is also

corroborated by evidence morphological indicating that the relationship between neuroblasts undifferentiated embryo- fetal and Schwann cells recapitulates not those commonly found between nerve cells neoplastics and Schwann cells in many areas of neuroblast of nodular type.

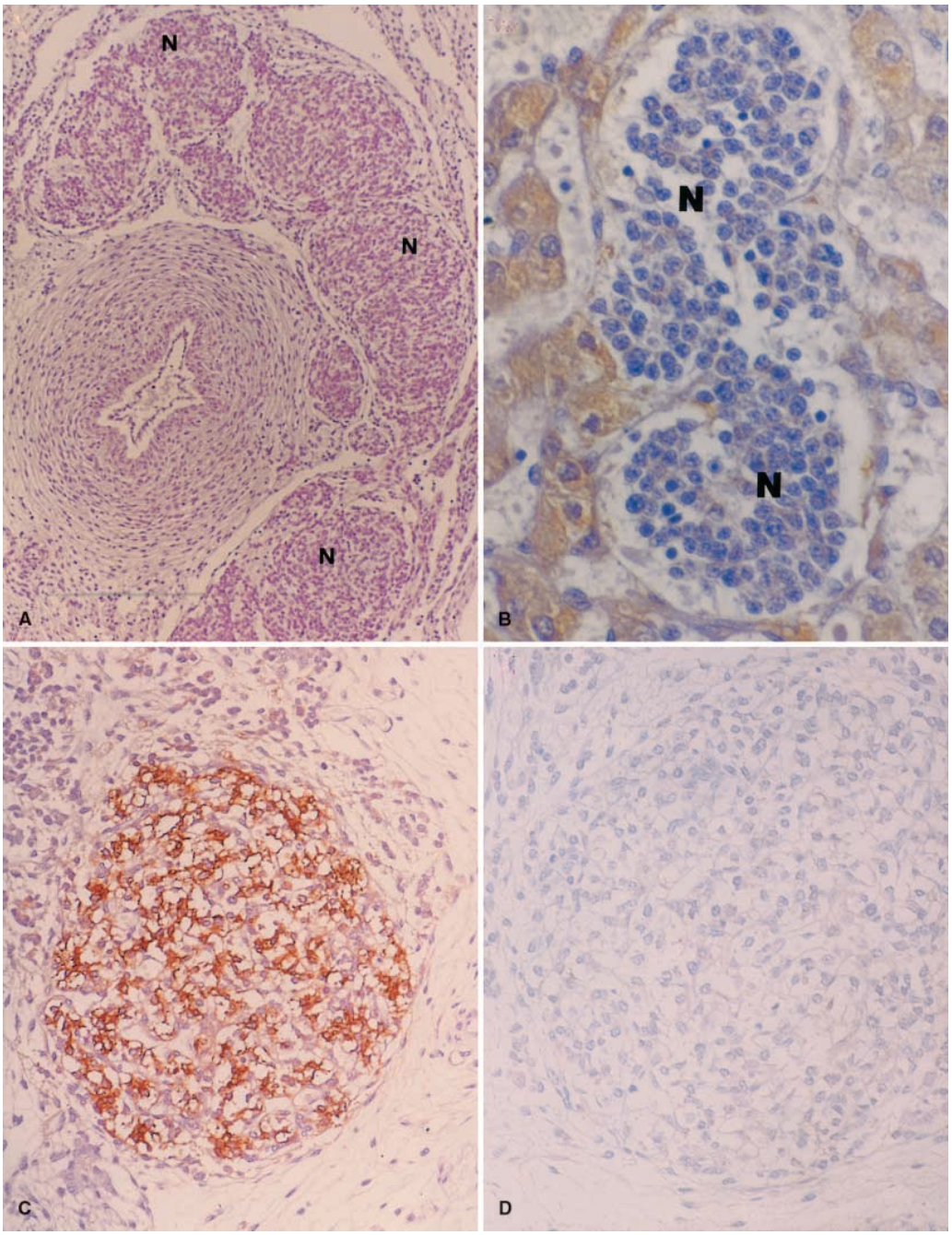
It is likely that the amount and distribution of cellular the glial cells in neuroblastic tumors (neuroblastoma, ganglion neuroblastoma, ganglioneuroma) can be submind determined by several environmental factors , including the production of growth factors by neuroblasts tumoral. It has been suggested that, for their part, the cells of Schwann, producing several trophic factors, such as the factor nerve growth (NGF), the factor –β maturation of glial (GMF β), brain-derived neurotrophic factor (BDNF), could contribute to differenziazione/maturation of neuroblastoma. Evidence in favor king of this possible role is the high expression of TrKA , receptor for NGF, in many cancers with favorable prognosis honourable. Future studies on the ontogeny of human SNOP, with particular emphasis on glial cells, could help to better understand the role that these cells play in the processes of migration, differentiation and maturation cellular, laying the foundation for a model of ontogenetic study of neuroblastoma.

1.10 Normal tissues

During the early phases of development (from the 8th to the 12th wGA), clusters of primitive sympathetic neuroblasts (round or oval cells with a tiny cytoplasmic rim and hyperchromatic nuclei with numerous nucleoli) interconnected by nerve fibres, were detected from the paravertebral regions to adrenals (Fig. 3A). These cell clusters colonised the adrenal glands and were found throughout the adrenal cortex to the central veins of the deep regions (Fig. 3B). From the 28th to the 38th wGA, these immature cell clusters progressively decreased in number until disappearance in neonatal adrenals. Throughout development, the neuroblasts were stained with NSE but did not show any CD immunoreactivity (Fig. 3A, B). From the 8th wGA within the immature neuroblastic cell clusters, some larger cells, most likely developing (immature) ganglion cells, were immunostained for CD (Fig. 4A). In older fetuses (from the 12th to the 38^{th} wGA), a steadily increasing granular to diffuse cytoplasmic staining for CD was detected in these developing ganglion cells in the preaortic, paravertebral and periadrenal ganglia,

in the adrenal medulla, and in submucosal and myoenteric nervous plexuses of the gastrointestinal tract (Fig.4B, C). The fully differentiating ganglion cells were recognisable for the progressive cell enlargement and the vesicular nucleus with one or more prominent nucleoli (Fig. 4B–D). CD immunoreactivity was maintained in ganglion cells of neonatal and adult sympathetic ganglia, adrenal glands, and gastrointestinal nervous plexuses. Schwann cells of nerve fibres associated with ganglion cells lacked any CD immunoreactivity (Fig. 4D). CD immunostaining was also detected in the cytoplasm of the developing adrenocortical cells (Fig. 3B) surrounding the clusters of undifferentiated neuroblasts, and it was maintained in neonatal and adult adrenal cortex. From the 8th wGA, differentiating adrenal and extraadrenal (sympathetic ganglia and paraganglia) chromaffin cells were identifiable for their chromogranin A immunoreactivity (Fig. 3C). In the adrenals, these cells were closely associated with the primitive neuroblastic cell clusters, as individual cells or small nests. They progressively increased in number and size from the 28th wGA to develop the adrenal medulla. Extra- and intraadrenal immature chromaffin cells were not stained with CD (Fig. 3C, D), while adult adrenal medullary chromaffin cells showed focal and weak CD immunoreactivity.

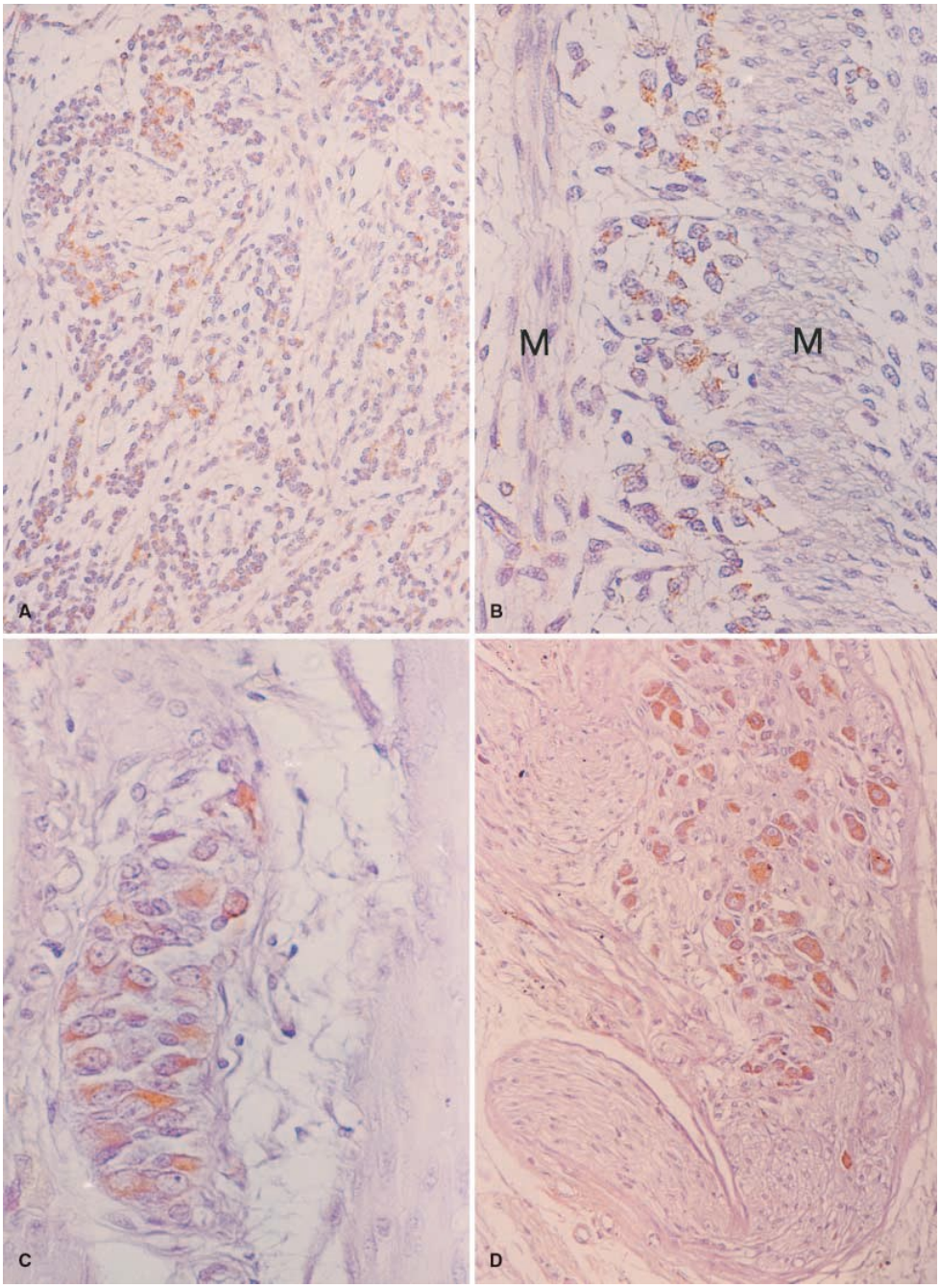
Fig. 3



Magro G et al., 2000

Fig. 3 Peri-preaortic (A) and intra-adrenal (B) clusters of undifferentiated neuroblasts (N), in human fetuses of 10 weeks and 12 weeks of gestational age (wGA), respectively, are not stained for cathepsin D (CD). Cytoplasmic immunoreactivity for CD is, however, shown by the adrenocortical cells (B) surrounding neuroblasts. A paravertebral paraganglion of a 15-wGA human fetus is stained with chromogranin A (C), but it is unreactive for CD (D) in consecutivesections. Original magnifications, A ×100; B ×250; C, D ×125

Fig.4



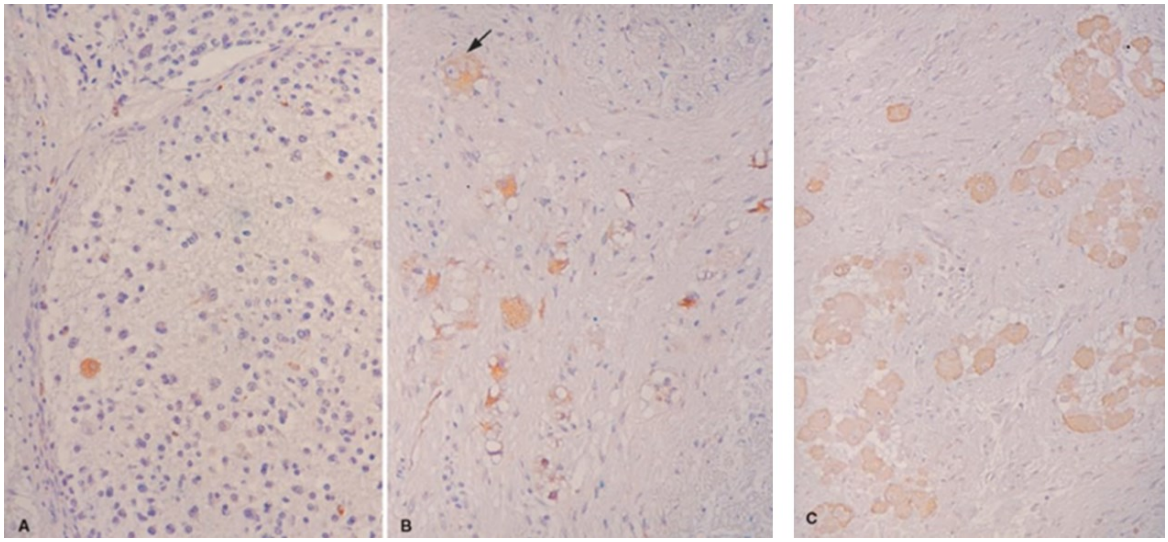
Magro G et al., 2000

Fig. 4 A Most cells of a paravertebral cluster of neuroblasts in a 12-week of gestational age (wGA) fetus exhibit cytoplasmic immunoreactivity for cathepsin D (CD). B Nervous cells with morphological features of immature ganglion cells and with a distribution within the smooth muscle layer (M) typical of the developing myoenteric nervous plexus are stained with CD in the rectum of a 15-wGA fetus. C Ganglion cells of the gastric myoenteric nervous plexus in a 34-wGA fetus show strong cytoplasmic CD immunoreactivity. D Ganglion cells of a peri-adrenal ganglion of a 36- wGA fetus are strongly positive for CD, whereas Schwann cells are unstained. Original magnifications, A ×125; B ×320; C ×400; D ×100

1.11 Peripheral neuroblastic tumours

The morphologically undifferentiated neuroblasts in neuroblastomas and ganglio-neuroblastomas did not exhibit any CD immunostaining (Fig. 5A). This was detected in the cytoplasm of neuroblasts showing morphological evidence of gangliocytic differentiation (cytoplasmic and nuclear enlargement, cytoplasmic eosinophilia, tumour giant cells with a single large or multiple nuclei; Fig. 5A, B), as well as in the ganglion cells of both ganglioneuroblastomas and ganglioneuromas (Fig. 5B, C). Supportive spindle cells surrounding tumour cell nests were also stained in some areas.

Fig.5



Magro G et al., 2000

Fig. 5A Differentiating neuroblastoma. Neuroblasts showing morphological features of gangliocytic differentiation are stained for cathepsin D (CD). In the undifferentiated neuroblasts, CD is absent or only focally expressed. B Ganglioneuroblastoma, borderline type. The depicted area is composed by rare mature ganglion cells (arrow) and neuroblastic cells showing a variable degree of ganglion cell differentiation (immature ganglion cells), interspersed within a Schwannian stroma. Both mature and differentiating ganglion cells show CD immunoreactivity, while Schwann cells do not. C Neoplastic ganglion cells of a ganglioneuroma are immunoreactive for CD. Original magnifications, A–C ×125

Several morphological, immunohistochemical and in vitro studies indicate that childhood NTs recapitulate the subsequent developmental stages of normal PSNS (Cooper MJ et al., 1990a, Cooper MJ et al., 1990b, Hoehner JC et al., 1996, Hoehner JC et al., 1998, Joshy VV et al, 1992, Kelly DR et al., 1996, Krajewski S et al., 1995, Tsokos M et al 1987). This has prompted the search for specific cell differentiation markers (Goji J et al., 1995, Hedborg F et al., 1995, Hoener JC et al., 1995, Hoehner JC et al., 1995, Hoehner JC et al., 1996, Krajewski S et al., 1995, Magro G et al., 1997, Magro et al., 1995, Molenaar et al., 1990) suitable for diagnostic purposes (Kelly DR et al., 1996) and for a better understanding of the biology of NTs (Hoehner JC et al., 1996, Hoehner JC et al., 1998). The focus on ganglion cell differentiation is of special interest because the extent of gangliocytic differentiation is one of the most reliable parameters for the classification and the prognostic evaluation of NTs (Joshy VV et al., 1994, Joshy VV et al., 1992, Kelly DR et al., 1996). Investigations on developing human PSNS and GENS, which arise from a common neural crest-derived precursor cell (Coupland RE et al., 1995, Gershon MD et al., 1993), allow the pathway of ganglion cell differentiation to be followed, and have shown that it is characterized by the appearance of a distinct immunophenotype (Hoehner JC et al., 1996, Hoehner JC et al., 1998). However, markers of ganglion cells are usually not specific to this cell lineage because they are also shared by chromaffin cells (tyrosine hydroxylase, CD44, NSE) and neuroblasts (neuropeptide-Y, HNK-1/N-CAM, Bcl-2) (Hoehner JC et al., 1996, Hoehner JC et al., 1998). Recently, CD has been immunolocalised in the intestinal ganglion cells of human neonates and adults, and it has been considered as a specific cell marker, suitable for diagnostic purposes in routinely processed tissues (Abbu-Alfa AK et al., 1997). We have investigated the developmentally regulated expression and distribution of CD in human PSNS and GENS, and compared the results with those obtained in childhood NTs. During PSNS and GENS development, CD immunoreactivity is restricted to ganglion cell lineage, whereas undifferentiated neuroblasts and developing chromaffin cells remain consistently unstained. CD immunoreactivity parallels the morphological differentiation of ganglion cells, as documented by a progressively more intense cytoplasmic staining of the developing ganglion cells with increasing gestational ages. CD immunoreactivity is also maintained in the ganglion cells of sympathetic ganglia and GENS in neonates and adults.

In infantile NTs, CD immunoreactivity is restricted to neuroblastic cells showing morphological evidence of ganglion cell differentiation (differentiating neuroblastomas, ganglioneuroblastomas) and to the mature ganglion cells of both ganglioneuroblastomas and ganglioneuromas. These findings confirm previous observations (Parham D et al., 1985) of CD immunoreactivity in neuroblasts showing gangliocytic differentiation, and in neoplastic ganglion cells of neuroblastomas/ganglioneuroblastomas and ganglioneuromas, respectively.

The comparative evaluation of the immunohistochemical findings in fetal and neoplastic tissues indicates that CD expression in childhood NTs mirrors its normal developmental regulation in PSNS, as already reported for Bcl-2, c-ErbB2, insulin-like growth factor 2 and β2-microglobulin (Cooper MJ et al., 1990, Goji J et al., 1995, Hedborg F et al., 1995, Krajewski S et al., 1995). This strongly supports the view that infantile NTs arise from a disturbed and/or blocked differentiation process at different stages of the PSNS ontogenesis (Cooper MJ et al., 1990, Hoehner JC et al., 1996, Hoehner JC et al., 1998, Joshy VV et al., 1992, Kelly DR et al., 1996, Trojanowski JQ et al., 1991). The role of CD expression in developing and mature ganglion cells and whether it is directly involved in ganglion cell differentiation remain to be elucidated.

In conclusion, although CD is widely expressed in a variety of normal and neoplastic human tissues, including the developing and mature adrenocortical cells as shown in the current study, this proteinase is a reliable ganglion cell differentiation marker in the human PSNS and GENS, as well as in childhood NTs. It may be particularly useful in the diagnosis of developmental abnormalities of the enteric nervous system (Hirschprung's disease and neuronal intestinal dysplasia), as previously suggested (Abu-Alfa AK et al., 1997), and in the assessment of the extent of gangliocytic differentiation in NTs.

ASSUMPTION

On the basis of previous results, illustrated in chapter 1, and contained mainly in the paper "*Glial cells in the ontogenesis of the human peripheral sympathetic nervous system and in neuroblastoma"* by Magro & Grasso (2000), the purpose of my research was aimed.

In particular, I focused on the analysis of a protein involved in the development of the peripheral nervous system: the *Wilms' tumor1* (WT1).

My thesis is divided into two experimental phases: I) spatio-temporal distribution of WT1 during human embryonic development, II) expression and functional roles of WT1 during development of the peripheral sympathetic nervous system and gastrointestinal tract.

The results obtained by these two experimental phases conduced to the following papers:

- Rosalba Parenti, Roberto Perris, Giada Maria Vecchio, Lucia Salvatorelli, Antonietta Torrisi, Lucia Gravina, Gaetano Magro "*Immunohistochemical expression of Wilms' tumor protein (WT1) in developing human epithelial and mesenchymal tissues.*" Acta Histochem. vol. 115, no. 1, pp. 70-5, 2013

- Rosalba Parenti, Lidia Puzzo, Giada Maria Vecchio, Lucia Gravina, Lucia Salvatorelli, Giuseppe Musumeci, Enrico Vasquez, Gaetano Magro "*Developmental expression of WT1 in human peripheral sympathetic and gastroenteric nervous system: an immunohistochemical study".* Acta Histochem. 2013 Jun 19. pii: S0065-1281(13)00091-3. doi: 10.1016/j.acthis.2013.05.003.

Chapter2

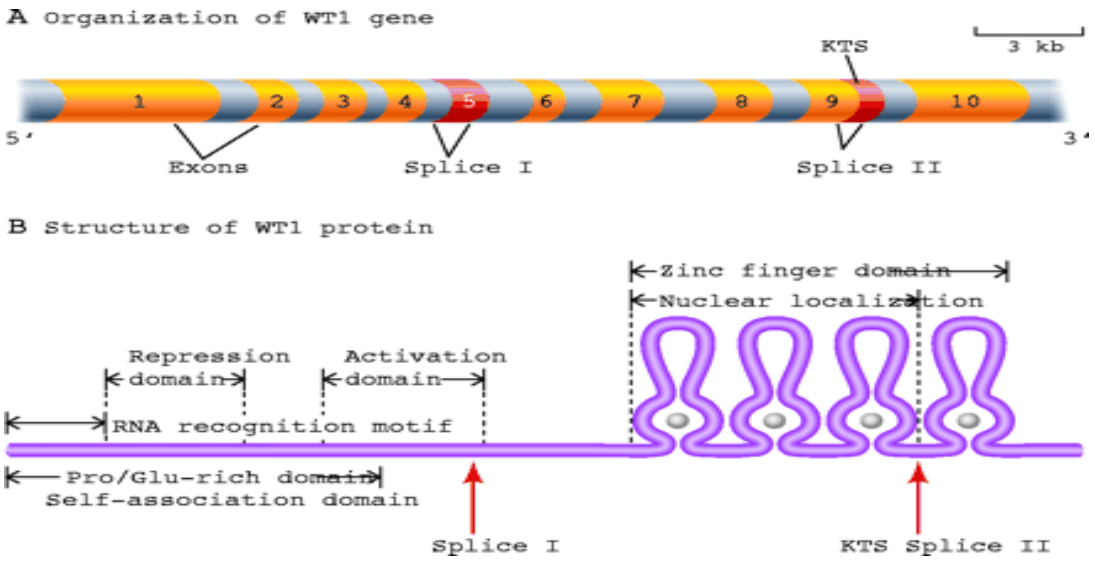
2.1 WT1 GENE AND PROTEINS

The human *WT1* gene spans ~50 kb and consists of 10 exons (Call KM et al., 19905, Gessler M et al., 1990) (Fig.6). It encodes a protein that shares a high degree of structural homology with the early growth response family of transcription factors (Rauscher FJ et al., 1993). The *WT1* gene product contains four COOH-terminal C2H2 zinc fingers for nucleic acid binding (Rauscher FJ et al., 1990). Its NH2 terminus includes both transcriptional repression and activation domains (Fig.6). Additional motifs in the WT1 protein are essential for self association, nuclear localization, and RNA recognition (Fig.6). More than 20 different *WT1* gene products with molecular masses of 52–65 kDa are generated by a combination of alternative mRNA splicing (Haber DA et al., 1991), initiation of translation at variable start codons (Bruening W et al., 1996), and RNA editing (Sharma PM et al., 1994). Among them, alternatively spliced exon 5 encodes 17 amino acids at a site NH2- terminal of the zinc finger domain (Haber DA et al.,1991). A second splicing event, which involves the use of two alternative splice donor sites at the end of exon 9, leads to the insertion/omission of three amino acids (lysine, threonine, and serine; KTS) between zinc fingers 3 and 4 of the WT1 molecule (Haber et al., 1991) (Fig.6). The corresponding proteins, which are designated as WT1 (–KTS) and WT1(+KTS), respectively, differ in their DNA binding site selectivity. Computer modeling (Kennedy D et al., 1996) and in vitro studies (Caricasole A et al., 1996) revealed a higher affinity for RNA of the +KTS proteins compared with the –KTS forms.

Furthermore, the WT1(+KTS) products colocalized with and bound to nuclear splicing factors (Davies RC et al., 1998, Englert C et al., 1995, Larsson SH et al., 1995). These findings strongly support the possibility that the WT1(+KTS) proteins play a role in mRNA splicing rather than transcriptional control.

The ratio of the +KTS and –KTS proteins is conserved among tissues (Haber DA et al., 1991), and imbalanced expression of both isoforms will lead to developmental abnormalities. In humans, a ~50% reduction of the WT1(+KTS)

levels due to heterozygous point mutation in a splice donor site in intron 9 was associated with developmental defects known as Frasier syndrome (Barbaux S et al., 1997). Frasier syndrome is characterized by severe glomerulopathy of the kidneys and male-tofemale sex reversal (female external genitalia, streak gonads, XY karyotype). In an attempt to define the molecular function of the WT1(+KTS) protein, transgenic mice were generated that only expressed the –KTS product. Animals with selective lack of the WT1(+KTS) form displayed a phenotype reminiscent of Frasier syndrome in humans (Hammes A et al., 2001). Malformations, i.e., hypoplastic kidneys and streak gonads, were even more severe in the WT1*(–KTS)* deficient mice (Hammes A et al., 2001). Hence, the functional complexity of WT1 appears to be determined by the generation of multiple protein forms.



Scholz H and Kirschner KM., 2005

Fig. 6 *Organization of the Wilms' tumor1 (WT1) locus and basic structure of the WT1 proteins The WT1 gene spans ~50 kb on human chromosome 11p13 and consists of 10 exons. Of particular interest are two alternative splicing events: Alternatively spliced exon 5 encodes 17 amino acids, and the use of an alternative splice donor site at the end of exon 9 leads to the insertion of three amino acids—lysine, threonine, and serine (KTS) between zinc fingers 3 and 4 of the WT1 protein. Whereas the WT1(–KTS) gene products act as transcriptional regulators, the +KTS forms might play a yet-undefined role in posttranscriptional mRNA processing. Additional WT1 molecules are generated by translation from variable start codons, RNA editing, and posttranslational modification.*

2.2 *STRUCTURE AND ALTERNATIVE SPLICING OF WT1*

Distinct WT1 Isoforms

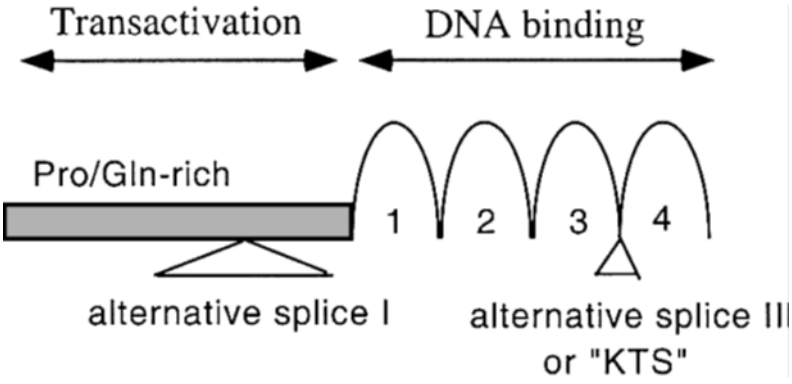
WT1 encodes a protein migrating around 50 kDa, which contains two domains with apparent functional properties: a C-terminal C2H2 zinc finger domain involved in DNA binding and an N-terminal proline/glutamine-rich transactivational domain (see Fig. 7).

The coding sequence is comprised of 10 exons, with each zinc finger encoded by an individual exon (Haber DA et al., 1991). Two alternative pre-mRNA splicing events give rise to distinct gene products. Exon 5 encodes 17 amino acids that are inserted between the transactivation and DNA-binding domains (alternative splice I). The 17 amino acids encoded by alternative splice I can mediate some transcriptional repression activity in a reporter assay, when fused to a heterologous DNA-binding domain (Wang Z et al., 1995), although similar transactivational properties are observed using native WT1 proteins containing or lacking this insertion (Madden S et al., 1991). The precise function of this 17-amino-acid insertion therefore remains uncertain. Alternative splice II results from the use of a variable splice donor site between exons 9 and 10, leading to the insertion of three amino acids, lysine–threonine-serine (commonly referred to as KTS), between the third and the fourth zinc fingers (Haber DA et al., 1991). This insertion disrupts the critical spacing between these zinc fingers resulting in loss of DNA binding to the consensus WT1 DNA-binding sequence (Rauscher FJ et al., 1990). This observation, and the fact that WT1(+KTS) proteins appear to be colocalized with elements of the pre-mRNA splicing machinery (Larsson SH et al., 1995), have led to the suggestion that this isoform plays a role in RNA processing rather than transcriptional activation. Whatever its function, the importance of the ratio between the (-KTS) and (+KTS) isoforms is highlighted by Frasier syndrome, a severe developmental defect affecting kidneys and gonads that has been linked to presence of a germline mutation in the exon 9 splice donor consensus, associated with reduced expression of WT1(+KTS).

Additional isoforms of WT1 have been reported, resulting from RNA editing (leucine to proline at codon 280) (Sharma PM et al., 1994) and use of an upstream CUG initiation codon (Bruening W et al., 1996), but the functional consequences

resulting from these differences remain undefined. Recently, a truncated form of WT1 has been detected in several cell lines and in Wilms' tumor specimens due to an internal initiation at an in-frame AUG codon at position 127, giving rise to a 36- to 38-kDa protein (Scharnhorst V et al., 1999). This internal initiation site, which is evolutionarily conserved, is also predicted to generate all four alternative splicing variants of WT1. Absence of the N-terminal domain may lead to enhancement of transcriptional activation over transcriptional repression, although the truncated products are expressed at considerably lower levels than the full-length protein, and their functional significance remains to be determined. Most studies of WT1 function have focused on the conventional AUG initiated transcripts encoding or lacking alternative splices I and II (KTS). Ectopic expression of constructs encoding the various isoforms of WT1 in a Wilms tumor cell line inhibits cellular proliferation, consistent with a tumor suppressor effect (Haber DA et al., 1993). However, it is possible that the development of physiologically relevant assays for WT1 function will bring insight into the relevance of these additional low abundance products.

Fig.7



Sean Bong Lee and Daniel A. Haber., 2001

***Fig.** 7 Schematic representation of WT1 gene product. The N-terminal transactivation domain and the C-terminal zinc finger domain are noted, along with the two alternative splicing events. Alternative splice I inserts 17 amino acids between the transactivation and DNA-binding domains; alternative splice II results from the use of an alternative splice donor site and introduces 3 amino acids (KTS) between zinc finger 3 and 4, altering the DNA-binding properties of the encoded protein.*

DNA-Binding Sequence

The four Kruppel-like C2H2 zinc fingers of WT1 mediate DNA binding by the (-KTS) isoform. Zinc fingers 2–4 have a high degree of amino acid homology to those of the immediate early gene *early growth response 1* (*EGR1*) (Sukhatme VP et al., 1988), although the three zinc fingers of *EGR1* are encoded by a single exon, a genomic structure that is distinct from that of *WT1* and suggests that these genes do not share a common evolutionary origin.

The WT1 zinc finger domain binds to the characteristic GC-rich EGR1 DNA-binding element, although with ~40-fold less affinity than EGR1 itself (Rauscher FJ et al., 1990, Drummond IA et al., 1994). Most WT1-responsive promoters identified to date contain one or more EGR1 sites within their promoters. A second potential DNA-binding site for WT1 consists of TCC repeats, which have been mapped by analysis of the WT1-responsive promoters of PDGF-A (Wang ZY et al., 1993) and epidermal growth factor receptor (EGFR) (Englert C et al., 1995). More recently, PCR selection of genomic DNA sequences with high affinity for the WT1(-KTS) zinc fingers, coupled with extensive mutational studies, led to the characterization of an optimized binding site, 59-GCGTGGGAGT-39 (Nakagama H et al., 1995). This binding site, called WTE, displays 20- to 30-fold higher affinity for WT1 than does the EGR1 sequence. This high-affinity WTE site has recently been reported to mediate binding by WT1(-KTS) to the promoters of Amphiregulin (Lee SB et al., 1999) and Bcl2 (Mayo MW et al., 1999), two genes that appears to be regulated by WT1 *in vivo.* Analysis of additional WT1(-KTS)-target genes will be required to determine whether presence of the WTE sequence within a promoter is more useful in predicting regulation of the native gene by WT1 than does presence of the EGR1 or TCC-repeat motifs. Some evidence suggests a potential DNA recognition sequences for WT1(+KTS), but the precise binding site remains uncertain (Bickmore WA et al., 1992, Little MH et al., 1996).

Transcriptional Repression

Transcriptional repression by WT1 was first demonstrated through fusion of the N-terminal domain to a heterologous DNA-binding domain (Madden SL et al., 1991). The observation that the WT1 (-KTS) isoform binds to the EGR1 DNA-binding consensus sequence subsequently made it possible to test the transactivational properties of the full-length protein using promoter–reporter assays.

The characterization of WT1 as a transcriptional repressor of genes containing the GC-rich EGR1 sequence within their promoter led to the identification of a large number of potential target genes, whose promoters are repressed by ectopic expression of WT1 in transient transfection assays (Rauscher F et al., 1993). These genes include EGR1 (Madden SL et al., 1991), WT1 itself (Rupprecht HD et al., 1994, Hewit SM et al., 1996), PDGF-A (Gashler AL et al., 1992, Wang ZY et al., 1992), IGF2 (Drummond IA et al., 1992), insulin-like growth factor receptor (IGFR) (Werner H et al., 1993, Werner H et al., 1995), Pax2 (Ryan G et al., 1995), colony stimulating factor 1 (CSF1) 1993), C-myc (Hewitt SM et al., 1995), N-myc (Zhang X et al., 1999), Bcl2 (Hewitt SM et al., 1995, Heckman C et al., 1997), Inhibin α (Hsu SY et al., 1995), G protein αi-2 (Kinane TB et al., 1995), and telomerase RT (TERT) (Oh S et al., 1999) among others. Some of these genes encode embryonic growth factors, providing an attractive hypothesis to explain the tumor suppressor properties of WT1, but most were identified by virtue of their GC-rich promoters, a relatively common characteristic. In fact, the generation of cells with inducible expression of WT1 isoforms demonstrated that induction of WT1(-KTS) was not accompanied by altered expression of the native genes, despite potent repression of corresponding promoter-reporter constructs in transfection assays (Englert C et al., 1995, Thate C et al 1998).

Transcriptional Activation

Like transcriptional repression, activation of promoter-reporters by WT1 has been described, an effect that appears to be modulated by both promoter and cellular contexts (Wang ZY et al., 1993, Reddy JC et al., 1995, Maheswaran S et al., 1993, Nichols KE et al., 1995). Recently, the physiological relevance of WT1-mediated

transcriptional activation has been supported by a number of observations: (1) induction of cell cycle arrest by WT1(-KTS) is linked to its induction of the cyclin dependent kinase inhibitor p21Cip1 (English MA et al., 1999, Englert C et al., 1997); (2) a Wilms-tumor-associated point mutation in WT1 abrogates transcriptional activation but not repression (English MA et al., 1999); (3) *in vivo* physical association of WT1 with the transcriptional coactivator CBP/P300.
Haber, unpublished]; and (4) use of expression profile analysis to identify endogenous genes, such as Amphiregulin that are transcriptionally induced by WT1(-KTS) (Lee SB et al., 1999). Ectopic expression of WT1(-KTS) in a number of cell types leads to a G1 cell cycle arrest, an observation that is correlated with induction of the endogenous p21Cip1 gene, and activation of its promoter in reporter assays (English MA et al., 1999, Kudoh T et al., 1995, Englert C et al., 1997). The promoter of Bcl-2 is either repressed or activated by WT1(-KTS), depending on cell type (Mayo MW et al., 1999, Hewitt SM et al., 1995, Heckman C et al., 1997). The Bcl-2 promoter contains the optimal WT1-binding site, WTE, and increased levels of endogenous Bcl-2 expression are observed in the rhabdoid tumor cell line G401, following stable transfection of WT1(-KTS) (Mayo MW et al., 1999). Induction of Bcl-2 by WT1 may contribute to its survival function in renal precursors. Syndecan-1 is a mesenchymal proteoglycan thought to play a role in epithelialization of the kidney (Vainio S et al., 1989). The Syndecan-1 promoter appears to be activated by both the (-KTS) and (+KTS) isoforms ofWT1 through a region that is GC rich, although the precise DNA sequences required for this effect remain undefined (Cook DM et al., 1996). E-cadherin is also a cell surface protein, which is expressed in the condensing metanephric mesenchyme. WT1(-KTS) mediates activation of the E-cadherin promoter and induction of the endogenous transcript is detectable following ectopic expression in NIH 3T3 cells (Hosono S et al., 2000). Loss of E-cadherin expression is common to many human cancers, although this has not been correlated with the presence of WT1 mutations in Wilms tumors.

2.3 FUNCTIONAL ROLES

WT1 as a regulator of transcription and post-transcriptional processes

The first function described for WT1 was a role in transcriptional regulation. As a recent review on this aspect of WT1 is available (Roberts SG., 2005), we can here be brief about this. Again, paradoxical findings have been described on the exact role of WT1 in this process, where it appears to function as either a transcriptional activator or repressor, depending on the cellular and experimental context. For many years, the identification of WT1 downstream targets solely relied on in vitro overexpression and reporter assay data, complicated by the fact that no clear and unique WT1-binding element has been found. Recent years have seen the publication of data on transcriptional WT1 targets that passed more tests for in vivo relevance, such as differential expression in WT1 mutant mouse models, interaction of endogenous WT1 with DNA detected via chromatin immunoprecipitation or overlapping expression patterns in mouse embryos. Maybe surprisingly, so far all these confirmed target genes such as Amphiregulin (Lee SB et al., 1999), Sprouty1 (Gross I et al., 2003), TrkB (Wagner N et al., 2005), nephrin (Wagner N et al., 2004), nestin (Wagner N et al., 2006) and Pou4f2 (Wagner KD et al., 2003) appear to be activated rather than repressed by WT1. Yet, several cofactors such as BASP1 (Carpenter B et al., 2004) and WTIP (Srichai MB et al., 2004) have now been described, which specifically act as transcriptional co-suppressors for WT1, confirming this is a physiological relevant function for WT1. It is important to add that only the -KTS isoforms, constituting around one-third of total WT1 protein, bind DNA
with high affinity and function efficiently in transcriptional regulation.

In addition to a role in transcriptional regulation, there is a wealth of circumstantial evidence pointing to a role for WT1 in RNA metabolism, possibly splicing, mediated via Zn-finger 1 and with some specificity for the -KTS isoform (Larsson SH et al., 1995, Ladomery M et al., 2003).

However, we await demonstration of a specific role of WT1 in RNA metabolism and its physiological relevance. In this regard, it will be important to identify whether WT1 binds to specific RNA molecules in vivo and whether mutation of WT1 leads to altered processing of these RNAs. Recent data suggest the role of

WT1 might not be limited to transcriptional regulation and RNA metabolism. Anecdotal evidence from many labs showed that some endogenous Wt1 protein could be found in the cytoplasm, but for a long time, this was dismissed as an antibody-staining artefact. It has now been found that, in fact, 10–25% of endogenous WT1 in murine kidney and differentiated ES cells is indeed cytoplasmic and shuttles actively between the nucleus and the cytoplasm (Niksic M et al., 2004). Even more surprising, fractionation of cytoplasmic protein showed that WT1 could be found at the actively translating polysomes. Although so far highly speculative, it does open up the possibility that WT1 might play a role in translation as well. If this is proven to be the case, it will be interesting to see whether the same genes that are transcriptional targets are also bound as RNA and during translation, in which case the function of WT1 might be rather different from what is believed at the moment.

Recently, the first study to report a specific post-transcriptional function for WT1 was published. The authors showed that +KTS but not -KTS WT1 isoforms can stimulate polysome binding and translation of an RNA retaining an intron (Bor YC et al., 2006). Although the physiological relevance of these findings remains to be confirmed, they demonstrate a new potential function for WT1.

Tumour suppressor gene versus oncogene

WT1 is widely accepted to function as a tumour suppressor gene in the formation of Wilms' tumours. However, over the past few years, data have accumulated on the expression of WT1 in adult tumours from different origin, including colorectal (Koesters R et al., 2004), breast (Loeb DM et al., 2001), desmoid (Amini N et al., 2005) and brain tumours (Oji Y et al., 2004).

As these tumours arise in tissues that normally do not express WT1 but no mutations in the gene have been identified, it has been suggested that expression of WT1 might play an oncogenic role in these tumours. In fact, evidence using antisense oligonucleotides shows that WT1 is required for proliferation while inhibiting apoptosis of tumour cells in culture (Tuna M et al., 2005). WT1 expression in the adult appears to be limited to the kidney podocytes; therefore, oncogenic WT1 expression might be a relatively tumour-specific target for therapeutic intervention. Indeed, trials using peptide vaccines against WT1 in patients with leukaemia, breast or lung cancer were promising (Oka Y et al.,

2004). If WT1 is functionally active in the tumourigenic process in these tumours, additional therapeutic schemes can be envisioned.

These observations will need further study. First of all, so far there are no clear data on the isoforms expressed in these tumours. As in vitro data on the isoforms that result from the alternative start sites (up- or downstream) suggest dominant-negative effects for these isoforms, this might be an important aspect of the role of WT1 in these tumours. Second, all publications on WT1 expression in adult cancers show mainly, if not only, cytoplasmic localization of the protein. This too might be part of an oncogenic role for WT1, as normally only 10–25% of the protein is found in the cytoplasm (Niksic M et al., 2004). Third and finally, it is not known whether WT1 is expressed during development of the tissues where the tumours are found. If so, the expression found in the tumours might reflect either de-differentiation of cells or the cancer stem cell origin of the tumour. New gain and loss-of-function mouse models will need to be developed to fully analyse the oncogenic potential of WT1 isoforms as oncogenes.

The apparent contrasting roles of WT1 in inducing differentiation versus inhibiting differentiation and the context-dependent bi-directional control of mesenchymal epithelial fate might partially explain how WT1 can function as a tumour suppressor gene in some tissues and as a potential oncogene in others. The adult cancers where WT1 is expressed are generally derived from epithelial cells.

It is clear that many inconsistencies with respect to WT1 still exist. However, there is the possibility that these inconsistencies might in fact begin to reveal the true functions of WT1 in normal development and disease.

Carpentieri's hypothesis that there is a role for WT1 in the pathogenesis of tumors with rhabdomyomatous differentiation and that the cytoplasmatic positivity for WT1 has to be interpreted as a special expression of this gene.

These results also suggest that WT1 plays a role in the chemotherapy activity on the blastemal component of the Wilms' tumors.

The Wilms' tumour protein (WT1) shuttles between nucleus and cytoplasm

The WT1 protein is predominantly nuclear as a transcriptional regulator. However, it has also become clear that the function of WT1 is more complex and that it is likely to be involved in at least two cellular processes: transcription control and RNA metabolism, depending on isoform differences (Davies R et al.,

1999, Hastie ND., 2001). The first connection between the WT1 protein and RNA metabolism was established by Larsson et al. (Larsson SH et al., 1995), who showed that the +KTS isoform co-localized preferentially with splicing factors within nuclear speckles. Soon after that Caricasole et al. (Caricasole A et al., 1996) presented data showing that RNAse but not DNAse treatment can impair its localization within the nucleus. A putative RNA recognition motif in amino acids 11–72 of WT1 has been identified (Kennedy D et al., 1996). The suggestion that WT1 is indeed involved in RNA processing and very likely in splicing was further supported by data showing that WT1 (+KTS more than -KTS) directly associates with the constitutive splicing factor U2AF65, which is part of the splicing machinery that recognizes the 30 splice site. However, U2AF65 binding is not needed for the speckled distribution of WT1, as shown by WT1 mutants unable to bind to U2AF65 that still co-localize with splicing factors in nuclear speckles (Davies RC et al., 1998). Another WT1 binding protein, WTAP, is also involved in splicing. WTAP is a homologue of the Drosophila protein FL(2)D that is required for female-specific splicing of Slx and Tra pre-mRNAs mediated by 30 splice site choice (Granadino B et al., 1996, Penalva LO et al., 2000). In vitro, WTAP can replace FL(2)D in the regulation of female splicing of Tra pre mRNA and is also found to be present in functional spliceosomes (Ortega A et al., 2003, Zhou Z et al., 1999). Moreover, the WT1–RNA metabolism connection was further strengthened by the observations that WT1 co-purifies with nuclear poly(A)+ ribonucleoproteins.

Interestingly, despite the suggestion of functional differences between the two isoforms (+/-KTS), both can co-sediment with RNPs on density gradients from fetal kidney cell line M15 nuclear extracts (Ladomery MR et al., 1999). Despite the accumulating evidence of WT1 involvement in RNA processing, it is still not clear what role WT1 plays in this cellular process nor have in vivo target RNAs been identified.

Caricasole et al. (Caricasole A et al., 1996) showed that both WT1 -KTS and WT1 +KTS can bind to exon 2 sequences of the murine IGF-II transcript and that this binding was mediated by zinc finger 1.

The importance of zinc finger 1 in RNA binding was also confirmed in vivo (Ladomery M et al., 2003). Over the past few years it has become apparent that a significant number of transcription and/or splicing factors shuttle to the cytoplasm

where they may acquire a new function. The cytoplasmic function attributed to shuttling proteins is mostly nucleo-cytoplasmic transport of mRNA or RNA stability. Interestingly, some of these proteins are found to be present in functional ribosomes, indicating their involvement in translation (Dreyfuss G et al., 2002).

It was reported that WT1 is a shuttling protein and that a significant fraction of endogenous WT1 protein is present in the cytoplasm. Both WT1 isoforms, -KTS and +KTS, shuttle between the nucleus and the cytoplasm. Furthermore, cytoplasmic WT1 is present within mRNP complexes, and subsequently is associated with ribosomes and actively translating polysomes. These new data strengthen the idea that WT1 plays a role in post-transcriptional processes and extend its potential range of functions in the cell.

Several RNA processing factors have been shown to shuttle continuously between the nucleus and the cytoplasm, which is suggestive of their involvement in cytoplasmic functions (Dreyfuss G et al., 1998). WT1's previous characterization showed it to be a nuclear protein that acts as a transcriptional regulator and also is implicated in mRNA metabolism.

The WT1 protein is an interesting example of a multifunctional protein whose nuclear function seems to be divided between two different isoforms, +KTS and -KTS. Despite their spatial and functional differences in the nucleus, the results from transfection experiments show that both isoforms can shuttle into the cytoplasm and both are found to be associated with functional polysomes. This result seems contradictory to the proposed different functions for the two isoforms. However, this is not unexpected when taking into consideration that these different isoforms show some redundancy at the biochemical and genetical level (Hastie ND., 2001).

It is possible that, instead, direct modifications to the WT1 protein itself trigger its dissociation from the RNA and export from the nucleus. For example, there is evidence that at least two serine residues within zinc fingers 2 and 3.

This could explain why not all cytoplasmic WT1 is in the complex with RNP, as well as the observations that in the sucrose gradients the WT1 signal in RNP fractions are weak and become stronger as RNP become part of translation machinery.

It has been previously shown that WT1 accumulates in the cytoplasm in tumours of different tissue origins, such as rhabdomyosarcomas (Carpentieri DF et al.,

2002), some breast cancers (Silberstein GB et al., 1997) and colorectal adenocarcinomas (Oji Y et al., 2003). It is believed that in these tumour types WT1 plays an oncogene-like role rather than functioning as a tumour suppressor gene. The data raise the possibility that the potential oncogenic role of WT1 could be in regulating translation rather than nuclear processes. It remains to be seen whether cytoplasmic WT1 in tumour cells is associated with polysomes. Furthermore, a mislocalization per se, rather than overexpression, might have an oncogenic potential. The association of WT1 with polysomes extends even further the potential roles of this protein.

Expression of the Wilms' Tumor Suppressor Gene WT1 during Mouse Embryogenesis

Interestingly, there is also WT1 expression in the central nervous system which localizes to the ependymal layer of the ventral aspect of the spinal cord.

Immunohistochemical analyses using a WT1 antibody show that the presence of WT1 protein coincides with mRNA expression in the developing kidney, testes, uterus, and spinal cord, consistent with a role for WT1 in controlling the expression of genes involved in mesenchymal differentiation.

WT1 can either repress or activate potential target genes based on the context of the responsive elements (Madden S et al., 1991, Wang Z et al., 1993).

The occurrence of Wilms' tumor in association with genital abnormalities in WAGR and Denys-Drash syndromes (nephropathy, Wilms' tumor, and pseudohermaphroditism) has provided evidence for the role of *WT1* in genitouninary development (Breslow N et al., 1982, Coppes M et al., 1989. Moreover, heterozygous germline deletions of *WT1* in individuals with urogenital abnormalities suggest that this system is very sensitive to *WT1* levels during development (Pelletier J et al., 1991, Coppes M et al., 1992).

A role for the *WT1* gene during nephrogenesis is further supported by the observations that *WT1* mRNA levels correlate with specific histopathological features of Wilms' tumor which resemble early morphological features of nephrogenesis (Huang A et al., 1990, Yeger H et al., 1992, Pritchard-Jones K et al., 1991). The expression pattern of *WT1* in genitourinary development has been examined in the human, the rat, and the mouse (Pritchard-Jones K et al., 1990, Sharma PM et al., 1992); these studies show that *WT1* gene expression is

restricted to the developing genitourinary system, spleen, mesothelium, dorsal mesentery of the intestines, muscle, and central nervous system. Although these studies suggest that *WT1* is a tissue specific gene with differential expression during certain stages of development, the characterization of expression of *WT1* during murine embryogenesis would provide a more complete picture of temporal and spatial expression. Moreover, to date, there is little information on WT1 protein expression in development. Thus, the characterization of the temporal and spatial expression of *WT1* in the developing murine embryo and correlation of mRNA expression with the presence of the WT1 protein is of fundamental importance for understanding the role that this gene plays in normal organogenesis.

2.4 WT1 EXPRESSION IN TUMORS

Sporadic Wilms Tumor

The Wilms' tumour gene (WT1) encodes a zinc-finger DNA-binding transcription factor playing complex roles in proliferation and apoptosis, depending on cellular context. Wilms tumor, or nephroblastoma, is a pediatric kidney cancer thought to originate from pluripotent embryonic renal precusors (Bennington J et al., 1975). It arises in 1/10,000 children, usually around age 5, although children with genetic predisposition may develop bilateral Wilms tumors by age 2. In very rare cases, Wilms tumor may also occur in adults, presumably arising from persistent embryonic rests. Wilms tumor itself is composed of distinct histological elements, and is often described as "triphasic," reflecting the presence of epithelial and stromal components, as well as "blastemal" or undifferentiated mesenchymal cell. However, so-called anaplastic Wilms tumor remains refractory to treatment, a characteristic that has been linked to the frequency of p53 mutations in this rare variant (Bardeesy N et al., 1994). The genetic abnormalities underlying most cases of Wilms tumor remain unknown, although three genes have been implicated in a subset of tumors. WT1, the subject of this review, is inactivated in 15% of sporadic Wilms tumors. Insulin-like growth factor 2 (IGF2), a gene that is normally imprinted and expressed only from the paternal allele, may show "relaxation of imprinting" and hence increased dosage resulting from biallelic

expression. Finally, constitutive activation of b-catenin, a component of the Wnt pathway implicated in renal differentiation, is observed in 15% of tumors.

This may result in part from the lethality of this cancer prior to the advent of modern chemotherapy, and in fact, cases with familial transmission of WT1 mutations may become more common with the curative treatment of mutation carriers. In addition, reduced fertility may be associated with genitourinary malformations that may be present in carriers of *WT1* mutations.

However, many children with bilateral Wilms tumor do not have a family history of cancer and presumably carry a *de novo* germline mutation in a tumor suppressor gene (Narod S et al., 1991). Only a subset of these have demonstrated germline mutations in *WT1,* pointing to additional genes that are likely to contribute to genetic susceptibility to Wilms tumor. One such gene may be on chromosome 17, a locus implicated in at least one large family associated with late-onset of Wilms tumor in the absence of genitourinary malformations (Rahman N et al., 1996).

Expression in human neural tumors

During early development, WT1 is expressed in the brain and spinal cord, however its role in the malignancies that affect these tissues has not been previously investigated.

Recently, several genes have been identified as *in vivo* candidate *WT1*-regulated genes. These include the *EGFR*, *IGF1R*, *BCL2*, amphiregulin, E-cadherin and ornithine decarboxylase (Werner H et al., 1993, Hosono S et al., 2000). WT1 can regulate transcription through both activator and suppressor functions and this is affected by the cellular environment in which it is expressed.

The phenotype of *WT1* null mice demonstrates that it plays a pivotal role in the development of the urogenital system WT1 is expressed in tissues of mesodermal origin during embryogenesis including the kidney, gonads, heart, mesothelium and spleen. In these tissues WT1 is thought to play a role in regulating the mesenchymal to epithelial transition. The precise nature of the role of WT1 in this process is however not known, although there is evidence that transactivation of the *WT1* target genes, amphiregulin and E-cadherin, may be involved (Lee SB et al., 1999, Hosono et al., 1993). Oncogenic *WT1* lesions have been identified in

tumors arising in tissues of mesodermal origin including the kidney (Wilms' tumor) (Little MH et al., 1992) gut-lining (Desmoplastic small round cell tumor) (Gerald WL et al., 1995) and lungs (mesothelioma) (Park S et al., 1997). In addition, germline heterozygous WT1 zinc-finger mutations occur in Denys Drash syndrome in which patients are predisposed to the development of both Wilms' tumor and gonadoblastoma (Little M et al., 1997).

WT1 is also expressed transiently in the mouse, in between (Park s et al., 1993) and (Kleihues P et al., 1993) dpc in ectodermally derived tissues including the ependymal cells of the spinal cord and the ependymal cells that line the fourth ventricle of the brain (Armstrong JF et al., 1992). This suggests that WT1 may also play a role in the development of neural structures. This is also supported by the observation that PC19 embryonal carcinoma cells induced to differentiate to glial and neuronal cells with retinoic acid, switch on WT1 expression in the nucleus of the differentiating cells (Scharnhorst V et al., 1997). WT1 expression has also been reported in human brain tumor cell lines (Menssen HD et al., 2000).

Tumors of neuroepithelial origin (gliomas) comprise the largest group (43%) of all pediatric brain tumors and are histologically divided into several categories. The most aggressive gliomas are the Stage 4 glioblastoma multiforme and Stage 3 anaplastic astrocytomas. Lower grade gliomas are classified as grade 1 and 2 astrocytomas. Other major classes of brain tumor include medulloblastoma (20%), a primitive neuroectodermal tumor that arises in the cerebellum and ependymoma (8%). cells (the ectodermal cells of the neural tube) that ultimately populate the sympathetic ganglia, adrenal medulla and other sites. *in situ* hybridization studies for the involvement of WT1 in the development of neural tissues during embryogenesis, as well as the evidence from *in vitro* studies indicating that the induction of WT1 expression is coincident with the differentiation of glial and neuronal cells, we hypothesized that WT1 may be involved in the molecular etiology of human neural tumors. To investigate this we have analyzed brain tumor specimens for both WT1 expression and mutation.

WT1 is expressed in several different types of neural tumor and at low levels in the human brain. A significant number of tumors did not express WT1, however the lack of evidence for loss of function mutations in expressing tumors suggests that loss of WT1 expression in these tumors is probably not a mechanism associated with tumor development. As It was founded, no correlation between the

presence of WT1 expression and tumor staging, that would be suggestive of the involvement of oncogenic WT1 expression in more aggressive, less differentiated tumors, our data do not support an oncogenic role for WT1 in neural tumors. only 1 of 5 glioblastoma tumor specimens expressed WT1, suggesting that WT1 expression is independent of the differentiation state of the astrocyte and that it's expression is not clinically relevant in glioblastoma. In conclusion, although WT1 is expressed in the brain and is also expressed at detectable levels in human neural tumors, our evidence suggests that it does not play a significant role in the molecular etiology of these tumors.

Hereditary predisposition to Wilms' tumor has been associated with mental retardation and bilateral aniridia, in addition to genitourinary abnormalities. This complex of disorders implies a role for the WT1 gene in the development of the iris and the nervous system and/or the interaction of the WT1 gene product with a cluster of other genes that mediate this effect.

Curiously, in every section of the embryonic spinal cord from 14-day gestation to 1-wk-old rat, we observed a high level of expression of WT1 in the ventral horn of the spinal cord. In an effort to understand the role of the WT1 gene in the nervous system, we studied its expression in the rat brain by in situ mRNA hybridization. WT1 mRNA expression was seen exclusively in the area postrema. This cell group is one of the brain's circumventricular organs, which lacks a blood-brain barrier to circulating macromolecules. In line with this, the area postrema has been implicated as a chemoreceptor trigger zone for emesis and as a central site of action of circulating angiotensin II. Newborn and adult rats show comparable patterns and levels of WT1 mRNA expression in the area postrema, suggesting a continuing role for this gene product throughout the lifetime of the rat.

WT-1 mRNA expression was found to be specific to this area of the brain, since other circumventricular organs, including the median eminence and subfornicai organ, displayed no detectable hybridization signal in our experiments. The remarkably circumscribed localization of WT-1 transcripts in a structure which serves to transduce blood-borne signals of renal origin (the renin-angiotensin cascade) suggests a possible role for WT1 in integrated central and peripheral cardiovascular control mechanisms.

Expression in the female gonad was linked to the different stages of granulosa cell development. In the male gonad, expression was restricted to Sertoli cells and their precursors, the embryonic tunica albuginea and the rete testis.

Chapter 3

Immunohistochemical expression of Wilms' tumor protein (WT1) in developing human epithelial and mesenchymal tissues.

PURPOSE OF THE RESEARCH 1

Despite the fact that it was originally identified as a tumor suppressor gene playing a key role in Wilms' tumor, there is increasing evidence of its involvement in proliferation and apoptosis, depending upon the cellular context (Menke et al., 1998; Lee and Haber, 2001; Hohenstein and Hastie, 2006; Roberts, 2006; Hartkamp and Roberts, 2008; Huff, 2011). A combination of alternative splicing with different post-transcriptional modifications is the basis of the existence of at least 36 isoforms (Lee and Haber, 2001; Ellisen, 2002; Hohenstein and Hastie, 2006), whose activities may explain the different and apparently opposing functions of the different isoforms.

Besides its complex role in tumorigenesis, WT1 is also necessary for normal embryogenesis as shown by embryonic lethality, loss of kidneys, inhibited gonad development and defects in various mesothelium-derived structures in WT1 null embryos (Kreidberg et al., 1993; Herzer et al., 1999; Moore et al., 1999; Wagner et al., 2002b, 2005a). In addition, genitourinary malformations have also been observed in hemizygosity for WT1 in humans, suggesting that a commensurate WT1 gene dosage is necessary for normal development (Pelletier et al., 1991). Tissue expression of WT1 during embryonic development has been examined in human, rat, and mouse (Sharma et al., 1992; Armstrong et al., 1993; Mundlos et al., 1993; Rackley et al., 1993; Kent et al., 1995; Ramani and Cowell, 1996; Charles et al., 1997; Herzer et al., 1999; Moore et al., 1999; Scholz and Kirschner, 2005). These studies have shown that WT1 gene expression is restricted to the developing genitourinary system, spleen, mesothelium, dorsal mesentery of the intestines, smooth, skeletal and cardiac muscle, adrenal gland and some areas of the central nervous system. WT1 protein expression usually parallels the gene expression in most of the above mentioned tissues (Rackley et al., 1993).

In humans, WT1 protein expression appears restricted to nuclei of some fetal tissues including kidneys, gonads and related ducts, spleen, bone marrow, lungs, heart and arteries, intestine, smooth muscle of ureter and bladder wall, skeletal muscle, choroid plexus of brain and spinal cord (Pritchard-Jones et al., 1990; Sharma et al., 1992; Armstrong et al., 1993; Mundlos et al., 1993; Ramani and Cowell, 1996; Charles et al., 1997; Dennis et al., 2002; Lovell et al., 2003). However some differences in the immunolocalization of the protein have been reported, especially for skeletal and smooth muscle, heart, and uterus (Kent et al., 1995; Ramani and Cowell, 1996; Charles et al., 1997). These immunohistochemical results have been obtained by using antibodies directed against the Cterminal portion the molecule (clone WT C19). With the advent of new available antibodies against the N-terminal portion of WT1 protein (clone WT 6F-H2), some authors obtained WT1 expression within the cytoplasm of normal and neoplastic tissues. In this regard, there is some evidence that WT1 is expressed in the cytoplasm of the endothelial cells of normal blood vessels (Wagner et al., 2002a, 2005b; Bisceglia et al., 2010) and in most vascular tumors (Wagner et al., 2008; Trindade et al., 2011) and rhabdomyosarcomas (Carpentieri et al., 2002; Bisceglia et al., 2011), including Wilms' tumors with rhabdomyosarcomatous differentiation (Bisceglia et al., 2009). Although this cytoplasmic expression has been a matter of debate and originally was interpreted as a cross-reactivity with an epitope unrelated to WT1 (Ramani and Cowell, 1996; Charles et al., 1997), in vitro studies, Western blot and molecular analyses have confirmed the specificity of the cytoplasmic immunoreactivity (Carpentieri et al., 2002; Lawley et al., 2005; Timár et al., 2005). To the best of our knowledge, cytoplasmic immunostaining of WT1 has not been reported in human developing tissues, except for liver and proximal tubules of the kidney in which the staining was interpreted to be non-specific (Ramani and Cowell, 1996). Therefore the current study was undertaken to ascertain whether the WT1 protein is expressed in the cytoplasm of developing human tissues.

MATERIALS and METHODS

Fetal tissues

Tissue samples were collected from 20 human fetuses ranging from week 7 to 24 of gestation obtained from legal abortions.

These tissues have been previously used for other published immunohistochemical studies with the approval of the appropriate ethical boards (Magro and Grasso, 1995; Magro et al., 1995, 2001).

Fetal developmental age was based on size, including crown–heel, crown–rump and heel–toe measurements (Magro and Grasso, 1995; Magro et al., 1995, 2001).

Immunohistochemical analyses

All tissue samples had been fixed in 10% neutral buffered formalin for 12 h and embedded in paraffin.

Four μm thick sections were cut, stained with hematoxylin and eosin (H&E) and checked histologically to exclude pathological changes.

Immunohistochemical analyses were performed using the standard streptavidin–biotin-labeling technique using the LSAB kit (Dako, Glostrup, Denmark) with appropriate positive and negative controls. Sections derived from paraffin embedded specimens were deparaffinized in xylene for 15 min, rehydrated, and treated with 3% H2O2 for 10 min to block endogenous peroxidase activity, followed by extensive rinsing in double-distilled water and further rinsing for 15 min in 0.01 M phosphate-buffered saline (PBS), pH 7.4. Deparaffinized sections were incubated with anti-WT1 antibody (clone WT 6F-H2) (Dako, Glostrup, Denmark). Microwave pretreatment was crucial to enhance the staining in all samples examined. Accordingly, all sections were pretreated with citrate buffer (pH 6.0) and exposed to radiation in a microwave oven.

To reduce the commonly seen non-specific immunoreactivity due to endogenous biotin, sections were pretreated with 10 mg/mL of ovalbumin in PBS followed by 0.2% biotin in PBS, each for 15 min at room temperature. Bound antibody was revealed by incubation with 3,3-diaminobenzidine (Sigma–Aldrich, St. Louis, MO, USA) in 0.01% H2O2 for 5 min at room temperature. Sections were then counterstained with hematoxylin, dehydrated, and mounted. Negative controls involving the omission of the primary antibody were included. With regard to

WT1 immunostaining, the percentage of positively stained cells was assessed by semi-quantitative optical analysis according to a four-tiered system (<1% positive cells, negative staining; 1–10% positive cells, focal staining; 11–50% positive cells, heterogeneous staining; >50%, diffuse staining). Staining intensity was graded into weak, moderate, or strong intensity.

RESULTS

Immunohistochemical results are summarized in Table 2. From gestational weeks 7 to 24, WT1 expression was found in several tissues in a nuclear or a cytoplasmic localization (Table 2).

Table 2. *Immunohistochemical expression and distribution of WT1 protein in developing human tissues*

Organs	Sites	Nuclear/Cytoplasmic staining
Metanephrons	Glomerular epithelium Tubular epithelium Blastema	+++/--- +--/--- +++/---
Mesonephrons	Glomerular epithelium Tubular epithelium Stromal cells	+++/--- ---/--- ++-/---
Gonads	Germinal epitheliun Sex cords Stroma	+++/--- +++/--- +++/---
Müllerian/Wolffian ducts	Epithelium Stroma	---/--- +++/---
Celomic-derived serosal surfaces (pleura, peritoneum, ect)	Mesothelial cells Mesenchymal submesothelial cells	+++/--- +++/---
Skeletal muscle		---/+++
Myocardium		---/+++
Endothelial cells of blood vessels		---/+++

Staining intensity was evaluated as follows: no staining (−−−); focal staining (+−−) with ≤10% of positive cells; heterogeneous staining (++−) with 10–50% of positive cells; diffuse staining (+++) with >50% of positive cells.

Nuclear expression was mainly detected in epithelial tissues of the urogenital tract. A strong and diffuse WT1 nuclear staining was observed in metanephric and mesonephric podocytes, in the parietal layer of the Bowman's capsule (Fig. 8A and B) and in developing sex cords (Fig. 8B). Notably, a similar WT1 expression was also found in the mesothelial cells of all celom-derived membranes such as the pleura, the peritoneum and the serosal surfaces covering the abdominal and pelvic visceral organs (stomach, small and large intestine; pancreas, uterus and ovaries; bladder) (Fig. 8B and C).

With regard to mesenchymal tissues, nuclear immunoreactivity for WT1 was detected in metanephric blastema (Fig. 8A), gonadic stroma (Fig. 8B) and mesenchymal cells surrounding Müllerian and Wolffian ducts (Fig. 8B).

It is noteworthy that also numerous submesothelial mesenchymal cells, especially in the peritoneum, showed a strong and diffuse nuclear staining similar to that seen in the overlying mesothelial cells (Fig. 8C).

A further unexpected finding was a strong and diffuse WT1 cytoplasmic expression in the developing skeletal (Fig. 8D) and cardiac muscle cells (Fig. 8E) throughout the gestational period that was examined. A moderate to strong staining intensity for WT1 was also diffusely observed in the cytoplasm of endothelial cells of blood vessels in all developing tissues (Fig. 8F).

A concurrent nuclear WT1 immunostaining was not found in skeletal/cardiac muscle cells or in endothelial cells.

Fig.8

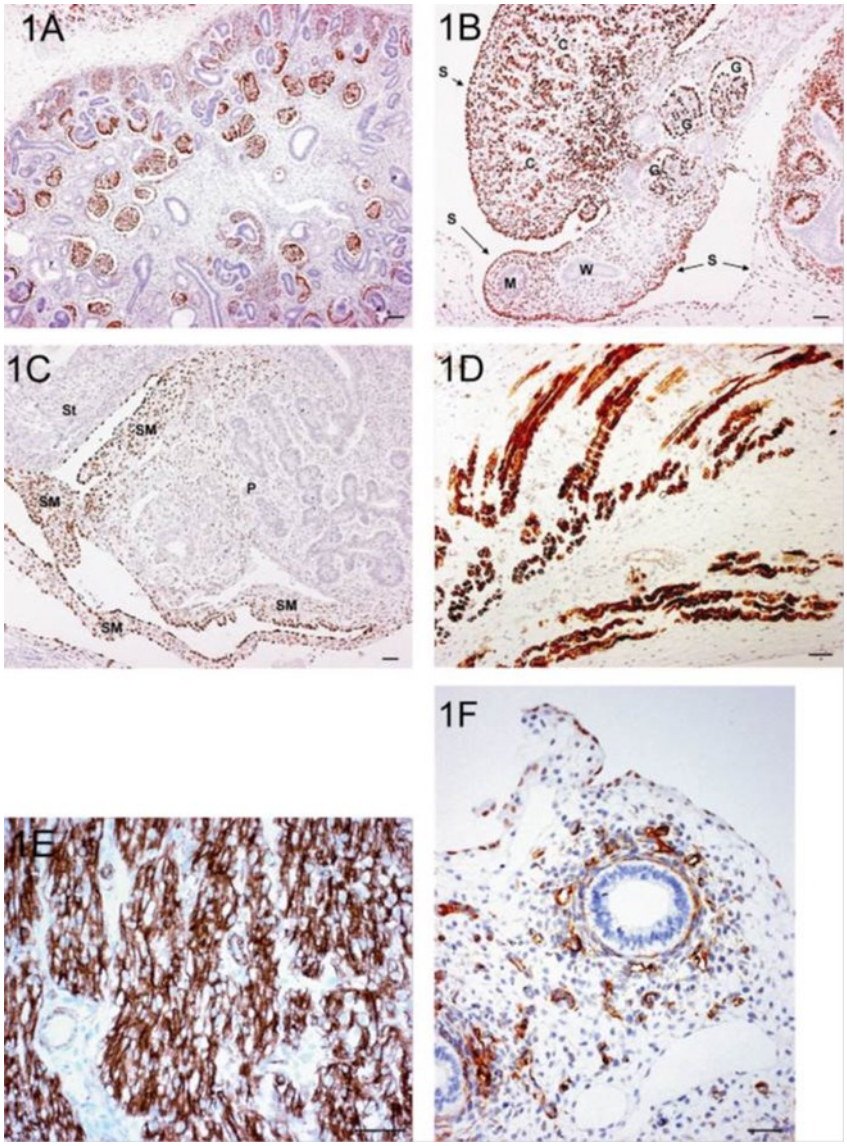


Fig. 8. *A. Metanephros from a human fetus of 11 weeks of gestational age. WT1 nuclear staining is observed in glomeruli and blastema. B. Human fetus of 10 weeks of gestational age. Mesonephric glomeruli (G) and developing sex cords (C) show nuclear WT1 expression. A similar staining can be observed in serosal surface epithelium (S) and mesenchyme surrounding Müllerian (M) and Wolffian (W) ducts. C. Human fetus of 10 weeks of gestational age. WT1 nuclear staining is observed in serosal surface epithelium covering pancreas (P) and stomach (St). Sub-mesothelial cells (SM) are stained similarly. D. Human fetus of 11 weeks of gestational age. A strong and diffuse WT1 staining is observed within the cytoplasm of skeletal muscle cells of the developing thoracic wall. E. Heart from a human fetus of 15 weeks of gestational age. Cardiac muscle cells (myocardium) exhibit a strong and diffuse cytoplasmic staining for WT1. No nuclear immunoreactivity is seen. F. Lung from a human fetus of 12 weeks gestational age.*
The cytoplasm of endothelial cells of developing blood vessels is stained with WT1. Nuclear immunoreactivity is seen in the serosal cells of visceral pleura. Scale bars = 100 _m.

The subcellular localization of the WT1 protein has been a matter of debate over the last two decades. The cytoplasmic immunoreactivity obtained by using some antibodies directed against the N-terminal portion of WT1 was originally questioned and judged to be due to cross-reactivities of these reagents or to correspond to non-specific staining caused by formalin-fixation as previously documented for other transcription factors such as c-myc gene product (Loke et al., 1988; Royds et al., 1992; Ramani and Cowell, 1996). Carpentieri et al. (2002) postulated that the WT1 cytoplasmic immunostaining detected in human rhabdomyosarcomas could be explained by the presence of an inactive form of the protein, which is activated by phosphorylation and translocated into the nucleus. In addition the different results, i.e. nuclear versus cytoplasmic WT1 localization reported in the literature are likely due to the different specificities of the antibodies used by the various authors. It should be emphasized that WT1 nuclear expression has been mainly observed by using antibodies directed against the C-terminal portion of the molecule (WT C-19 polyclonal antibody), while an exclusive cytoplasmic expression or coincident cytoplasmic and nuclear expression has been noticed with more recently generated available antibodies against the N-terminal portion (clone 6F-H2). Currently WT1 cytoplasmic localization is widely accepted as being a true localization of the molecule (Lawley et al., 2005; Timár et al., 2005; Nakatsuka et al., 2006; Tsuta et al., 2009). This is in line with WT1 involvement, not only in transcriptional regulation in the nucleus, but also in RNA metabolism and translational regulation in the cytoplasm as well as to nucleocytoplasmic shuttling properties of WT1 and its association with actively translating polysomes (Niksic et al., 2004).

In the present study we confirm the presence of a nuclear WT1 expression in selected developing epithelial tissues of the human fetus, consistently with previous reports in mouse, rat and man (Pritchard-Jones et al., 1990; Sharma et al., 1992; Armstrong et al., 1993; Mundlos et al., 1993; Rackley et al., 1993; Kent et al., 1995; Ramani and Cowell, 1996; Charles et al., 1997; Herzer et al., 1999; Moore et al., 1999; Dennis et al., 2002; Lovell et al., 2003; Scholz and Kirschner, 2005).

WT1 was mainly expressed in the nuclei of the urogenital tissues and mesothelial cells. In fact, both metanephric and mesonephric glomeruli, as well as developing sex cords, showed a strong and diffuse nuclear immunoreactivity. Apart from

epithelial cells, a nuclear staining was also obtained in cells located in the peri-Müllerian and peri-Wolffian mesenchyme and in the stroma of developing gonads. Notably, we observed that a significant number of mesenchymal submesothelial cells also expressed WT1 at the nuclear level, as did the overlying mesothelial cells. The close contact of the submesothelial cells with the overlying mesothelial cells would indicate a migration of the latter into the underlying mesenchyme, as previously proposed by other studies using antibodies against cytokeratins and extracellular matrix components (Magro et al., 1995, 2001; Magro and Grasso, 1995). Our results are consistent with the concept that human celomic epithelial cells contribute, at least in part, to the formation of submesothelial mesenchyme, especially of the abdominal and pelvic peritoneum. These findings may also explain the expression of WT1 in many human mesothelial-derived tumors, such as mesothelioma (Kumar-Singh et al., 1997; Nakatsuka et al., 2006), ovarian tumors (Shimizu et al., 2000; Zhang et al., 2003; Zhao et al., 2007) and Sertoli cell tumors (Zhao et al., 2007). Some authors have previously reported a strong cytoplasmic staining in human rhabdomyosarcoma (Carpentieri et al., 2002; Bisceglia et al., 2011), a tumor composed of malignant mesenchymal cells showing morphological, immunohistochemical and ultrastructural features of skeletal muscle differentiation, and in rhabdomyomatous Wilms' tumor (Bisceglia et al., 2009). The authors excluded the possibility of WT1 expression in developing skeletal muscle of an 8-weeks' gestation fetus. This led them to suggest that WT1 is a gene playing a role in the pathogenesis of rhabdomyosarcoma (Carpentieri et al., 2002; Bisceglia et al., 2011). Accordingly, we specifically focused on the developmentally regulated expression and distribution of WT1 in developing mesenchymal cells. The most striking finding was the immunodetection of WT1 in the cytoplasm of both developing skeletal and cardiac muscle cells in all the embryos and fetuses (gestational age of 7–24 weeks) included in this study. The early detection of WT1 in the cytoplasm of cells composing the above tissues would suggest that this transcription factor may undergo nuclear-cytoplasmic shuttling, acting as complex regulator of transcriptional/translational patterns during ontogenesis of both skeletal and cardiac muscle cells. The comparative evaluation of the immunohistochemical findings obtained here in fetal tissues with those of authors examining neoplastic tissues (Carpentieri et al., 2002; Bisceglia et al.,

2011) indicates that WT1 cytoplasmic expression in rhabdomyosarcoma mirrors its normal developmental regulation. Apart from the skeletal/cardiac muscle cells, we observed WT1 cytoplasmic expression also in endothelial cells of developing blood vessels. Interestingly, WT1 cytoplasmic expression has been documented in reparative neoangiogensis and in most benign and malignant vascular tumors (Al Dhaybi et al., 2010; Trindade et al., 2011). These findings are also in line with the proposed WT1 involvement in tumor vascularization where it may participate in the regulation of endothelial cell proliferation and migration (Wagner et al., 2008). In conclusion, although WT1 protein is widely expressed in a variety of developing human epithelial and mesenchymal cells, this transcription factor may be exploitable as a reliable marker of developing skeletal/cardiac muscle cells, as well as endothelial cells. Our findings support the rationale for the use of antibodies against the N-terminal portion of WT1 for diagnosis of both rhabdomyosarcoma and vascular tumors.

Chapter 4

Immunolocalization of Wilms' Tumor protein (WT1) in developing humanperipheral sympathetic and gastroenteric nervous system

PURPOSE OF THE RESEARCH 2

The Wilms' tumor gene (WT1) encodes a zinc-finger DNA-binding transcription factor playing complex roles in proliferation and apoptosis, depending on cellular context (Lee and Haber, 2001;Ellisen, 2002; Hohenstein and Hastie, 2006; Hartkamp and Roberts,2008; Huff, 2011; Parenti et al., 2013). Although it was originally shown that WTI played a central role in the formation of the genitourinary system (Call et al., 1990; Gessler et al., 1990;Pritchard-Jones et al., 1990; Menke et al., 1998), there is increasing evidence in support of the view that its developmental dynamic nuclear and cytoplasmic expression is crucial for the correct formation of several other organs (Scholz and Kirschner, 2005; Roberts,2006; Parenti et al., 2013). In this regard, WT1 protein expression appears restricted to nuclei of some fetal tissues including kidneys, gonads and related ducts, mesothelium, spleen, celomatic-derived membranes, bone marrow, lungs, heart and arteries, intestine, smooth muscle of ureter and bladder wall, skeletal muscle, choroid plexus of brain and spinal cord (Pritchard-Jones et al., 1990; Sharmaet al., 1992; Armstrong et al., 1993; Mundlos et al., 1993; Ramaniand Cowell, 1996; Charles et al., 1997; Dennis et al., 2002; Parenti et al., 2013). With the advent of new available antibodies against the N-terminal portion of WT1 protein (clone WT 6F-H2), some authors identified WT1 expression within the cytoplasm of fetal, adult and neoplastic endothelial cells (Wagner et al., 2002a, 2005a,2008; Bisceglia et al., 2010; Trindade et al., 2011; Parenti et al.,2013), and in the cytoplasm of fetal and neoplastic skeletal muscle cells (Carpentieri et al., 2002; Fraternali-Orcioni et al., 2010;Bisceglia et al., 2011; Salvatorelli et al., 2011).As far as WT1 expression in normal and pathological nervous tissues is concerned, there are only limited reported data in the literature. In situ hybridization analysis in mouse, showed a transient WT1 expression in

ependymal cells of the spinal cord and fourth ventricle of the brain (Sharma et al., 1992; Armstrong et al.,1993; Rackley et al., 1993; Dennis et al., 2002). In addition a potential role of WT1 in developing neural tissue seems to be demonstrated by the finding that WT1-null mice fail to form retinal ganglia (Wagner et al., 2002b) and olfactory epithelia (Wagneret al., 2005b). WT1 expression has also been reported in various neuroepithelial tumors (Dennis et al., 2002; Nakahara et al., 2004;Oji et al., 2004; Nakatsuka et al., 2006; Schittenhelm et al., 2009),such as gliomas (Schittenhelm et al., 2009; Bourne et al., 2010) and peripheral nerve sheath tumors (neurofibromas and schwan-nomas) (Schittenhelm et al., 2010). Apart from neoplastic tissues, WT1 involvement has also been proposed in neuro-degenerative disorders, such as Alzheimer's disease (Lovell et al., 2003) and Huntington's disease (Becanovic et al., 2010).Recently, we have found that WT1 is not only expressed at nuclear level of human fetal tissues, but also in the cytoplasm of skeletal muscle cells, cardiac muscle cells and endothelial cells(Salvatorelli et al., 2011; Parenti et al., 2013). This differential expression indicates that this protein may have shuttling properties, acting as a protein with complex regulator activity intranscriptional/translation processes during human ontogenesis(Parenti et al., 2013). These unexpected findings prompted us to investigate whether WT1 is expressed in the developing human peripheral sympathetic nervous system (PSNS) and gastro-enteric nervous system (GENS). This intriguing basic model involves ganglion and chromaffin cells differentiating from a common neural crest-derived cell precursor (Coupland, 1989; Gershon et al., 1993;Brodeur, 2003). To the best of our knowledge, there are no published studies on WT1 expression and distribution in the human developing PSNS and GENS.

MATERIALS and METHODS

Fetal tissues

We investigated the sympathetic neuroblasts and their derived-immature ganglion and chromaffin cells in: I) paravertebral, pre- and peri-aortic ganglia and paraganglia; II) adrenal glands; III) myoenteric nervous plexuses of gastro-intestinal tract (stomach; small and large bowel). Tissue samples were selected from paraffin-embedded blocks available from the files of the Anatomic Pathology Section of G.F. Ingrassia Department, University of Catania. Tissues were collected from 20 human fetuses ranging from the 8th to the 28th week of gestational age (wGA), obtained from legal interruptions or autoptic specimen. Fetal developmental age was based on size, including crown-heel, crown-rump and heel-toe measurements. The above mentioned tissues have been previously used for other published immunohistochemical studies with the approval of the appropriate ethical boards and in accordance with the Declaration of Helsinki (1964) (Parenti R et al., 2013, Magro G et al., 1995, Magro G et al., 2001). Sections from paraffin-embedded tissues were cut, stained with haematoxylin and eosin and checked histologically to exclude pathological changes.

Adult tissues

Seven normal adult adrenal glands with periadrenal sympathetic ganglia were obtained from patients who underwent radical nephrectomy for renal cell carcinoma. Myoenteric nervous plexuses were obtained from ten total gastrectomies and ten colorectal resections (including terminal ileum in two cases) of patients affected by invasive carcinoma. All tissue samples had been fixed in 10% neutral buffered formalin for 12 h and embedded in paraffin. Four μm thick sections were cut, stained with hematoxylin and eosin (H&E) and checked histologically to exclude pathological changes.

Immunohistochemical analyses

Immunohistochemical analyses were performed as previously reported in detail (Magro et al., 2011). Briefly, after appropriate deparaffinization and pre-treatments, sections were incubated with anti-WT1 antibody (clone WT 6F-H2 from Dako, Glostrup,Denmark) raised against the amino terminus portion of WT1, which is able to detect both cytoplasmic and nuclear staining (Parenti et al., 2013). Tissue sections from fetal adrenal glands were also incubated with anti-chromogranin A antibody (Dako, Glostrup,Denmark). Antigen retrieval with microwave pretreatment was crucial to enhance the staining in all samples examined. Accordingly, all sections were pretreated with citrate buffer (pH 6.0) and exposed to radiation in a microwave oven. To reduce the commonly seen non-specific immune reactivity due to endogenous biotin, sections were pretreated with 10 mg/mL of ovalbumin in PBS followed by 0.2% biotin in PBS, each for 15 min at room temperature. Bound antibody was revealed by incubation with 3,3-diaminobenzidine(DAB; Sigma–Aldrich, St. Louis, MO, USA) in 0.01% H2O2for 5 min at room temperature. Sections were then counterstained with hematoxylin, dehydrated, and mounted. Negative controls (omission of the primary antibody) were also performed.

RESULTS

Neuroblastic cell lineage

During the phases of development studied (from the 8th to the 28wGA), sympathetic neuroblasts, namely round- to oval-shaped cells with a thin rim of cytoplasm and hyperchromatic nuclei with small nucleoli, were found from the paravertebral regions to the adrenal glands and within the muscle wall of the developing stomach and small/large bowel. These cells formed round- to oval-shaped clusters which were arranged in the primitive paravertebral, pre- and para-aortic, peri- and intra-adrenal (from the cortex to the central veins of the deep portion of the gland) sympathetic ganglia and in the primitive myoenteric nervous plexuses of the gastro-intestinal tract. Fine nerve fibers interconnected the adjacent clusters of sympathetic neuroblasts along their course and intertwined among the neuroblasts. Despite the gestational age of the human foetuses, the neuroblasts of PSNS and GENS exhibited a strong and diffuse cytoplasmic positivity for WT1 (Figure 9A-D). WT1 also highlighted neuropil among the

sympathetic neuroblasts , that was not easily identified at light microscopic examination alone (Figure 9A). Interestingly, Schwann cells of the interconnecting nerve fibers showed a weaker cytoplasmic staining (Figure 9B,D). The cytoplasm of endothelial cells of blood vessels was strongly stained with WT1, and served as internal control. WT1 nuclear expression was not found in the sympathetic neuroblasts at any stage of development investigated.

Ganglionic cell lineage

From the 9th wGA, immature ganglion cells could be identified within the developing sympathetic ganglia and myoenteric nervous plexuses (Figure 10A,B). These cells, larger than neuroblasts, exhibited abundant eosinophilic cytoplasm and eccentrically located nuclei with one prominent nucleolus. Immature ganglion cells, in contrast to neuroblasts, showed a weak and focal to absent WT1 cytoplasmic staining (Figure 10A,B). Schwann cells of interconnecting nerve fibers were also stained with WT1 (Figure 10A). WT1 was not expressed in the ganglion cells of adult sympathetic ganglia, adrenal glands and myoenteric nervous plexuses. WT1 nuclear expression was not found either in the ganglion cells at any stage of development investigated or in the adult ganglion cells (not shown).

Chromaffin cell lineage

From the 8th wGA, differentiating chromaffin cells were identifiable as single cells, closely intermingling with sympathetic neuroblasts, within the developing extra- and intra-adrenal sympathetic ganglia. In addition they formed paraganglia, namely small cell clusters closely adjacent to ganglia and adrenal medulla. The latter was better appreciated from the 15th wGA and consisted of closely packed clusters of differentiating chromaffin cells in close proximity to the central veins of the deep portion of the adrenal gland (Figure 10C). Despite the gestational age of the human foetuses, the differentiating chromaffin cells were strongly and diffusely stained with chromogranin A (Figure 10C). Extra- and intra-adrenal differentiating chromaffin cells were not stained with WT1 (Figure 10D). Similar results were obtained in adult adrenal medulla and paraganglia. WT1 nuclear staining was not found either in differentiating or adult chromaffin cells (not shown).

Fig.9

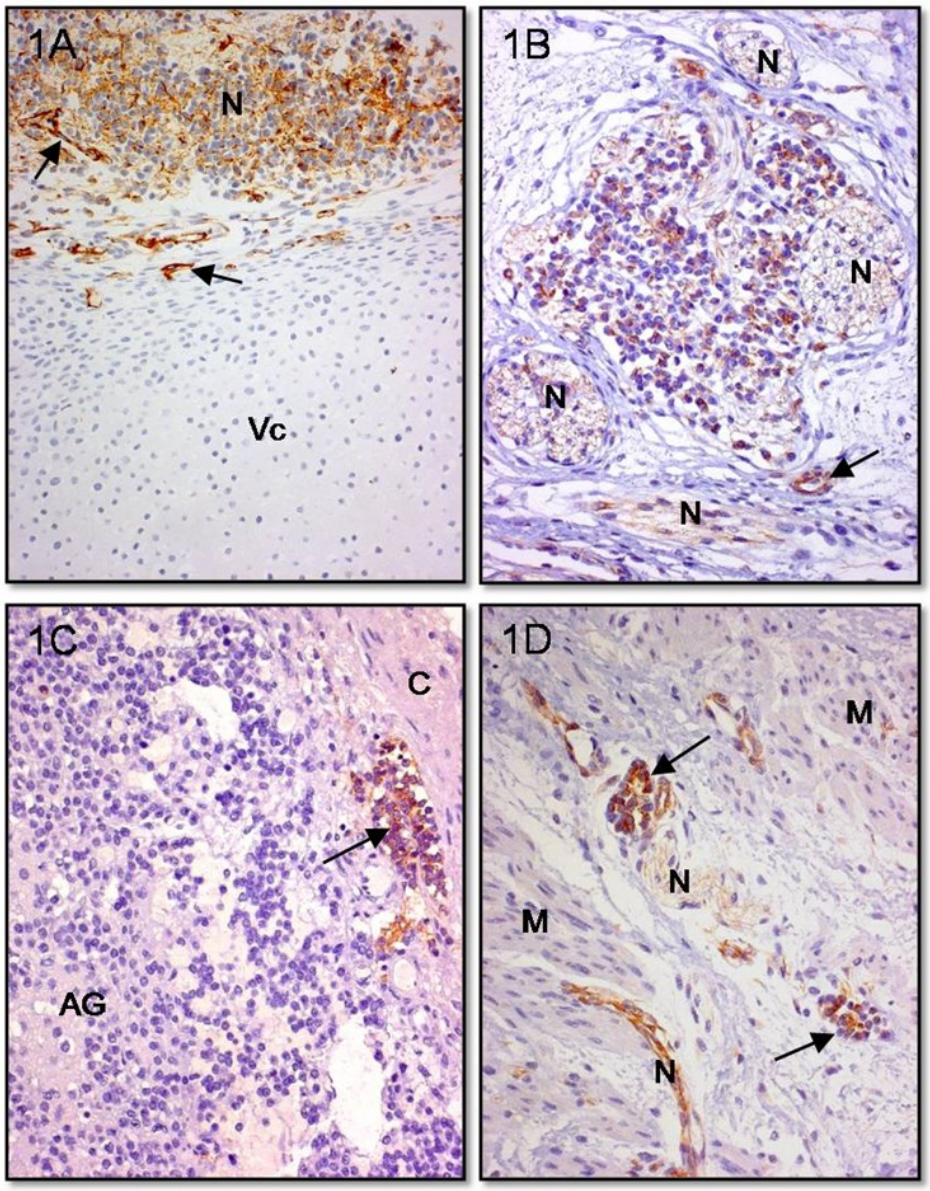


Fig. 9 *(**A**) Paravertebral cluster of sympathetic neuroblasts from a human fetus of 10 weeks of gestational age. WT1 stains the cytoplasm of neuroblasts and neuropil (N) among these cells. The cytoplasm of endothelial cells (arrows) is strongly stained with WT1 and served as internal positive control. Vertebral column (Vc) is unstained. Original magnification: x 150.*
*(**B**) Peri-aortic cluster of sympathetic neuroblasts from a human fetus of 11 weeks of gestational age. WT1 staining is observed in the cytoplasm of neuroblasts. Schwann cells of associated nerves (N), exhibit a weaker cytoplasmic staining. The cytoplasm of endothelial cells (arrow) is strongly stained with WT1 and served as internal positive control. (**C**) Human fetus of 12 weeks of gestational age. A small cluster of sympathetic neuroblasts (arrow), highlighted by WT1 cytoplasmic staining, is invading the capsule (C) of adrenal gland (AG). Original magnification: x 150. (**D**) Muscular wall (M) of stomach from a human fetus of 14 weeks of gestational age. WT1 expression is restricted to the cytoplasm of neuroblasts (arrows) of developing myoenteric nervous plexuses and Schwann cells of relative interconnecting nerves (N). Original magnifications: x100.*

Fig.10

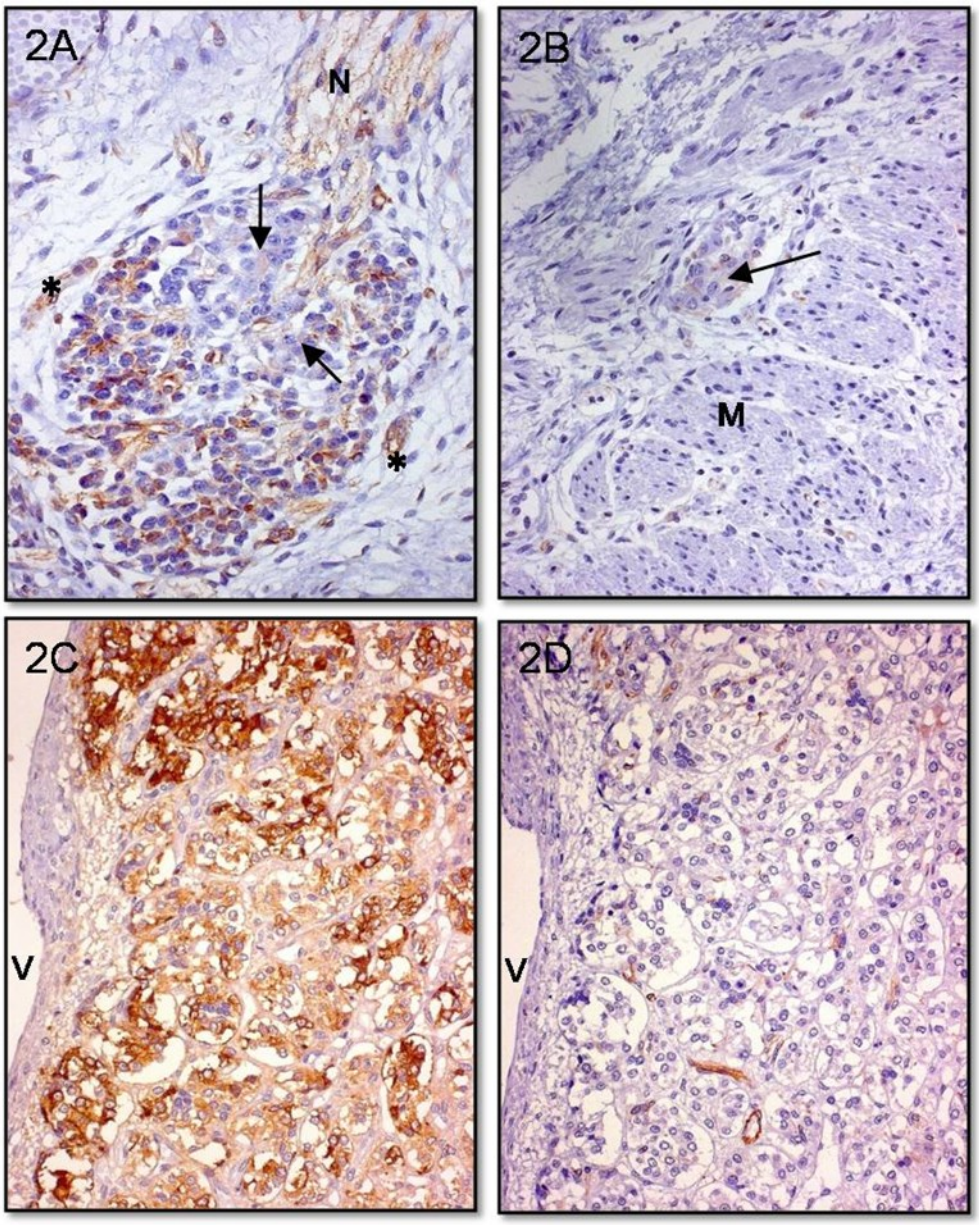


Figure 10 (A) *Human fetus of 14 weeks of gestational age. A paravertebral, developing sympathetic ganglion. Neuroblasts show a strong cytoplasmic staining for WT1. Immature ganglion cells (arrow) showing a weak to absent cytoplasmic WT1 expression. Schwann cells of associated nerves (N) and endothelial cells (asterisks) are also stained. (**B**) Muscular wall (M) of stomach from a human fetus of 19 weeks of gestational age. Morphologically immature ganglion cells of a myoenteric nervous plexus (arrow) are negative or only focally and weakly positive for WT1. (**C,D**) Serial sections of developing adrenal gland medulla from a human fetus of 20 weeks of gestational age. Differentiating chromaffin cells are strongly stained with cromogranin A (**C**), while they are negative for WT1 (**D**). V (central vein). The cytoplasm of endothelial cells are strongly stained with WT1 and served as internal positive control. Original magnifications: x100.*

DISCUSSION

The study of the developing PSNS and GENS is intriguing because it allows the possibility of following the bipotential capacity of a neural crest-derived precursor cell, namely sympathetic neuroblast, to mature either along a neuronal (ganglionic) or a neuroendocrine (chromaffin) lineage. Previous immune histochemical studies have shown that these sympathetic neuroblasts usually express neuron-specific enolase (NSE), tyrosine hydroxy-lase (TH), chromogranin A (CgrA), neurofilament proteins (NFs),CD44, Bcl-2 protein, HNK-1/carbohydrate epitope, endothelin-B receptor (ET-B), insulin growth factor-II (IGF-II) and hypoxia-inducible factor 2A (HIF2A) (Molenaar et al., 1990; Hedborg et al.,1995; Hoehner et al., 1996, 1998; Magro and Grasso, 2001; DePreter et al., 2007; Hoshi et al., 2009; Mohlin et al., 2013). Most of these markers are not specific, being maintained also in immature ganglion or chromaffin cells (Molenaar et al., 1990; Hedborget al., 1995; Hoehner et al., 1996, 1998; Magro and Grasso, 2001;De Preter et al., 2007; Hoshi et al., 2009; Mohlin et al., 2013).Indeed, the morphologic differentiation into ganglion or chromaffin cells is regulated by higher levels of expression of some of these neuroblast-associated markers, combined with concurrent loss of others. In particular, the ganglionic cell immunopheno-type is characterized by abundant expression of Bcl-2 protein,HNK-1/carbohydrate epitope, NFs, ET-B, cathepsin D, growth associated protein-43 (GAP-43) and by the lack of TH, CgrA and IGF-II(Hedborg et al., 1995; Magro and Grasso, 2001; De Preter et al.,2007; Salvatorelli et al., 2011; Mohlin et al., 2013). In contrast, TH, CgrA and IGF-II expression is pronounced in chromaffin cells, whileBcl-2 protein, HNK-1/ carbohydrate epitope, cathepsin D and GAP-43 are lacking (Hedborg et al., 1995; Magro and Grasso, 2001; De Preter et al., 2007; Salvatorelli et al., 2011; Mohlin et al., 2013).All these observations indicate that both chromaffin and ganglion cells establish their immune phenotype during the early phase of development. We first show that WT1 is transiently expressed in the cytoplasm of neuroblasts during human PSNS and GENS development. Unlike other neuroblast-associated markers (Hedborg et al., 1995;Magro and Grasso, 2001; De Preter et al., 2007; Mohlin et al.,2013), WT1 progressively disappears with advancing morphologic differentiation along both ganglion and chromaffin cell lineages. Accordingly, we suggest that WT1, when evaluated in the appropriate morphological context, is a

reliable marker of sympathetic neuroblasts of human PSNS and GENS, which can be used routinely in formalin-fixed tissues. WT1 cytoplasmic expression in human sympathetic neuroblasts, albeit unexpected, is not all that surprising. Although it was originally believed that WT1 was mainly expressed in the nuclei of some fetal tissues, including urogenital system-associated organs and mesothelial cells, we have recently reported its expression also in the cytoplasm of developing human skeletal/cardiac muscle cells and in endothelial cells of blood vessels (Salvatorelli et al., 2011; Parenti et al., 2013). A similar expression was documented in neoplastic cells of rhabdomyosarcoma, a malignant tumor showing skeletal muscle differentiation, and of several vascular tumors with endothelial differentiation (Carpentieri et al., 2002; Oue et al., 2011; Salvatorelli et al., 2011). All these data suggest that WT1 cytoplasmic expression in the neoplastic tissues parallels that observed in the developing counterpart tissues (Salvatorelli et al., 2011; Parenti et al., 2013). Based on the comparative evaluation of WT1 cytoplasmic expression in fetal, adult and neoplastic (rhabdomyosarcoma) skeletal muscle cells, we have suggested that this transcription factor can also be considered an oncofetal protein (Salvatorelli et al., 2011; Parenti et al.,2013). Apart from developing sympathetic neuroblasts, we first identified WT1 expression also in the cytoplasm of Schwann cells of the nerve fibers interconnecting the clusters of neuroblasts of human PSNS and GENS. This finding provides an explanation regarding the immunohistochemical expression of this marker in the cytoplasm of Schwann cells of human normal peripheral nerves and in benign and malignant peripheral nerve sheath tumors (Inagaki et al., 2011). The role of WT1 in developing PSNS and GENS is still to be elucidated. The variable WT1 expression and distribution at nuclear or cytoplasmic level, suggest different roles in the different human fetal tissues (Parenti et al., 2013). Its transient expression in sympathetic neuroblasts, associated with progressive loss in ganglion and chromaffin cells, would suggest that cytoplasmic retention of this protein may act by blocking differentiation processes, at least, during the early phases of development. WT1 cytoplasmic localization in human fetal neuroblasts, skeletal/cardiac muscle cells and in endothelial cells, is in line with its involvement not only in nuclear transcriptional regulation, but also in cytoplasmic RNA metabolism and translational regulation acting through nucleo-cytoplasmic shuttling properties (Niksic et al.,

2004).Several studies suggest that peripheral neuroblastic tumors in children, including neuroblastoma, arise from a disturbed and/or blocked differentiation process at different stages of PSNS development (Cooper et al., 1990a,b; Trojanowski et al., 1991; Joshyand Silverman, 1994; Hoehner et al., 1996; Magro and Grasso,2001; Brodeur, 2003). The evidence that the expression of several markers, including Bcl-2, c-ErbB2, insulin-like growth factor2 and b2-microglobulin (Hedborg et al., 1995; Cooper et al., 1990b;Goji et al., 1995; Krajewski et al., 1995), in childhood neuroblastic tumors recapitulates that observed during normal PSNS develop-ment seems to support this hypothesis. Whether WT1 expression in these tumors mirrors its normal developmental regulation remains to be established. However an immunohistochemical study on a relatively small series of peripheral neuroblastic tumors, including both neuroblastomas and ganglioneuromas, showed that ganglionic cells exhibited a higher expression of WT1 protein than undifferentiated neuroblastic cells (Wang et al., 2011). In contrast to what is observed in fetal tissues, WT1 protein may be a potential candidate for inducing primitive neuroblastic cells to differentiate into mature ganglion cells (Wang et al., 2011).

CONCLUSIONS

The principal conclusions of the experimental analyses performed in my thesis are the following:

1. Although WT1 protein is widely expressed in a variety of developing human epithelial and mesenchymal cells, it may be exploitable as a reliable marker of developing skeletal/cardiac muscle cells, as well as endothelial cells. The results support the rationale for the use of antibodies against the N-terminal portion of WT1 for diagnosis of both rhabdomyosarcoma and vascular tumors.
2. In contrast to what is observed in fetal tissues, WT1 protein may be a potential candidate for inducing primitive neuroblastic cells to differentiate into mature ganglion cells, according to recently reported by Wang (et al., 2011). A better understanding of the genes and proteins which regulate human PSNS development will help in the identification of the molecular pathways that are dysfunctional in pediatric neuroblastic tumors, providing model systems for target therapy.

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